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(54) Title: ANTI HIV COMPOSITIONS COMPRISING IMMUNOSTIMULATORY POLYNUCLEOTIDES AND HIV ANTIGENS			
(57) Abstract			
<p>The invention relates to anti-viral immunomodulatory compositions comprising immunostimulatory polynucleotides and HIV antigens, such as gp120. Methods for modulating an immune response upon administration of the oligonucleotide and antigen compositions are also disclosed.</p>			

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## ANTI HIV COMPOSITIONS COMPRISING IMMUNOSTIMULATORY POLYNUCLEOTIDES AND HIV ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the priority benefit of U.S. Provisional application 60/103,733, filed October 9, 1998, which is hereby incorporated herein by reference in its entirety.

TECHNICAL FIELD

10 The present invention relates to immunomodulatory compositions comprising a viral antigen, particularly an HIV antigen, and further comprising an immunostimulatory polynucleotide sequence (ISS). The invention further relates to the administration of the compositions to modulate an immune response.

15 BACKGROUND ART

In spite of recent development of a number of treatments, such as protease cocktails, infection by human immunodeficiency virus (HIV) remains an extremely serious health threat worldwide. HIV infection causes depletion of the subset of T cells called T helper cells (or CD4<sup>+</sup> cells), which in turn severely compromises the immune system, causing AIDS (acquired immune deficiency syndrome). Individuals suffering from AIDS are susceptible to a host of pathogenic infections and cancer.

20 The type of immune response generated to infection or other antigenic challenge can generally be distinguished by the subset of T helper (Th) cells involved in the response. The Th1 subset is responsible for classical cell-mediated functions such as delayed-type hypersensitivity and activation of cytotoxic T lymphocytes (CTLs), whereas the Th2 subset 25 functions more effectively as a helper for B-cell activation. The type of immune response to an antigen is generally determined by the cytokines produced by the cells responding to the antigen. Differences in the cytokines secreted by Th1 and Th2 cells are believed to reflect different biological functions of these two subsets.

30 The Th1 subset may be particularly suited to respond to viral infections and intracellular pathogens because it secretes IL-2 and IFN- $\gamma$ , which activate CTLs. The Th2

subset may be more suited to respond to free-living bacteria and helminthic parasites and may mediate allergic reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation, respectively. In general, Th1 and Th2 cells secrete distinct patterns of cytokines and so one type of response can moderate the activity of the other 5 type of response. A shift in the Th1/Th2 balance can result in an allergic response, for example, or, alternatively, in an increased CTL response.

Immunization of a host animal against a particular antigen has been accomplished traditionally by repeatedly vaccinating the host with an immunogenic form of the antigen. While most current vaccines elicit effective humoral (antibody, or "Th2-type") responses, 10 they fail to elicit cellular responses (in particular, major histocompatibility complex (MHC) class I-restricted CTL, or "Th1-type" responses) which are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, Th2-type responses are of little protective value against infection. Proposed vaccines using small peptides derived from the target antigen and other currently used antigenic agents that avoid use of potentially 15 infective intact viral particles, do not always elicit the immune response necessary to achieve a therapeutic effect. The lack of a therapeutically effective human immunodeficiency virus (HIV) vaccine is an unfortunate example of this failure. A recent review on the development of an HIV-1 vaccine suggested that for an HIV-1 vaccine to be effective, it must stimulate HIV-1-specific CTLs. Letvin (1998) *Science* 280:1875-1880.

20 Protein-based vaccines typically induce Th2-type immune responses, characterized by high titers of neutralizing antibodies but without significant cell-mediated immunity. In contrast, intradermal delivery of "naked", or uncomplexed, DNA encoding an antigen stimulates immune responses to the antigen with a Th1-type bias, characterized by the expansion of CD4<sup>+</sup> T cells producing IFN- $\gamma$  and cytotoxic CD8<sup>+</sup> T cells. Manickan et al. 25 (1995) *J. Immunol.* 155:250-265; Xiang et al. (1995) *Immunity* 2:129-135; Raz et al. (1995) *Proc. Natl. Acad. Sci. USA* 93:5141-5145; and Briode et al. (1997) *J. Allergy Clin. Immunol.* 99:s129. Injection of antigen-encoding naked DNA reproducibly induces both humoral and cellular immune responses against the encoded antigens. Pardoll and Beckerleg (1995) *Immunity* 3:165-169. DNA vaccines can provide a new approach to 30 infectious disease prophylaxis. See, for instance, Dixon (1995) *Bio/Technology* 13:420 and references cited therein.

Certain types of DNA, without being translated, have been shown to stimulate immune responses. Bacterial DNA induces anti-DNA antibodies in injected mice, as well as cytokine production by macrophage and natural killer (NK) cells. Pisetsky (1996a) *J. Immunol.* 156:421-423; Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Yamamoto et al. (1992a) *Microbiol. Immunol.* 36:983-897; and Cowdery et al. (1996) *J. Immunol.* 156:4570-4575.

B cell and NK cell activation properties of bacterial DNA have been associated with short (6 base pair hexamer) sequences that include a central unmethylated CpG dinucleotide. Yamamoto et al. (1992a); and Krieg et al. (1995) *Nature* 374:546-549.

Oligonucleotides comprising a CpG sequence flanked by two 5' purines and two 3' pyrimidines have been shown to be most potent in B cell and NK cell stimulation. For example, when a variety of oligonucleotides comprising hexamers were tested for their ability to augment the NK cell activity of mouse spleen cells, the most immunogenic hexamers included AACGTT, AGCGCT, GACGTC. Yamamoto et al. (1992b) *J. Immunol.* 148:4072-4076. In a study in which B cell activation was measured in response to oligonucleotides, the most stimulatory hexamer sequences (e.g., AACGTC, AACGTT, GACGTC, GACGTT) also matched the sequence of 5'-purine, purine, CG, pyrimidine, pyrimidine-3'. Krieg et al. (1995). However, as shown herein, this prototypical hexamer sequence is found in many oligonucleotides that are not immunostimulatory. Thus, the prototypical hexamer sequence proposed by Krieg et al. (1995) is not predictive of immunostimulatory activity.

Bacterial DNA stimulated macrophages to produce IL-12 and TNF- $\alpha$ . These macrophage-produced cytokines were found to induce the production of IL-12 and IFN- $\gamma$  from splenocytes. Halpern et al. (1996) *Cell. Immunol.* 167:72-78. *In vitro* treatment of splenocytes with either bacterial DNA or CpG containing oligonucleotides induced the production of IL-6, IL-12 and IFN- $\gamma$ . Klinman et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:2879-2883. Production of all of these cytokines is indicative of induction of a Th1-type immune response rather than a Th2-type response.

To date, no clear consensus has been reached on the sequences both necessary and sufficient of immune stimulation. A recent study which examined induction of NK activity in response to CpG containing-oligonucleotides suggested that the unmethylated CpG motif was necessary but not sufficient for oligonucleotide induction of NK lytic activity. Ballas

et al. (1996) *J. Immunol.* 157:1840-1845. Sequences flanking the CpG appeared to influence the immunostimulatory activity of an oligonucleotide. Immunostimulatory activity of immunostimulatory sequences appears to be independent of adenosine-methylation, and whether the nucleotide is single or double-stranded. See, for example, 5 Tokunaga et al. (1989) *Microbiol. Immunol.* 33:929; Tokunaga et al. (1992) *Microbiol. Immunol.* 36:55-66; Yamamoto et al. (1992b); Messina et al. (1993) *Cell. Immunol.* 147:148-157; and Sato et al. (1996) *Science* 273:352-354. Oligonucleotide length also does not seem to be a factor, as double-stranded DNA 4 kb long (Sato et al. (1996)) or single-stranded DNA as short as 15 nucleotides in length (Ballas et al. (1996)) elicited immune 10 responses; though if oligonucleotide length was reduced below 8 bases or if the DNA was methylated with CpG methylase, immunostimulatory activity was abolished. Krieg et al. (1995).

Vaccination with certain DNA containing immunostimulatory motifs induces an 15 immune response with a Th1-type bias. For example, mice injected intradermally with *Escherichia coli* (*E. coli*)  $\beta$ -galactosidase ( $\beta$ -Gal) in saline or in the adjuvant alum responded by producing specific IgG1 and IgE antibodies, and CD4 $^{+}$  cells that secreted IL-4 and IL-5, but not IFN- $\gamma$ , demonstrating that the T cells were predominantly of the Th2 subset. However, mice injected intradermally (or with a tyne skin scratch applicator) with 20 plasmid DNA (in saline) encoding  $\beta$ -Gal and containing an ISS responded by producing IgG2a antibodies and CD4 $^{+}$  cells that secreted IFN- $\gamma$ , but not IL-4 and IL-5, demonstrating that the T cells were predominantly of the Th1 subset. Moreover, specific IgE production by the plasmid DNA-injected mice was reduced 66-75%. Raz et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:5141-5145. In general, the response to naked DNA immunization is characterized by production of IL-2, TNF $\alpha$  and IFN- $\gamma$  by antigen-stimulated CD4 $^{+}$  T cells, 25 which is indicative of a Th1-type response. This is particularly important in treatment of allergy and asthma as shown by the decreased IgE production.

In another example, the presence of an immunostimulatory sequence, such as the palindromic hexamer AACGTT, in an antigen-encoding plasmid vector injected 30 intradermally prompted the production of large amounts of IFN- $\alpha$ , IFN- $\beta$  and IL-12. Sato et al. (1996). IFN- $\alpha$  plays a role in the differentiation of naive T cells toward a Th1-type phenotype, antagonizes Th2 cells, inhibits IgE synthesis, promotes IgG2a production and

induces a Th1 phenotype of antigen-specific T cell clones. IL-12 promotes IFN- $\gamma$  production by T cells and favors maturation of Th1 cells.

It would be useful in treatment of a wide variety of indications to be able to specifically enhance the Th1-type response to a particular antigen while down-regulating the Th2-type response to the same antigen. Treatment or palliation of these indications includes, but is not limited to, induction of a vigorous cellular immune response and anti-viral therapy. The present invention provides compositions comprising oligonucleotide sequences that can be employed in these contexts.

Other references describing ISS include: Krieg et al. (1989) *J. Immunol.* 143:2448-2451; Tokunaga et al. (1992); Kataoka et al. (1992) *Jpn. J. Cancer Res.* 83:244-247; Yamamoto et al. (1992b); Mojck et al. (1993) *Clin. Immuno. and Immunopathol.* 67:130-136; Branda et al. (1993) *Biochem. Pharmacol.* 45:2037-2043; Pisetsky et al. (1994) *Life Sci.* 54(2):101-107; Yamamoto et al. (1994a) *Antisense Research and Development.* 4:119-122; Yamamoto et al. (1994b) *Jpn. J. Cancer Res.* 85:775-779; Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523; Kimura et al. (1994) *J. Biochem. (Tokyo)* 116:991-994; Krieg et al. (1995); Pisetsky et al. (1995) *Ann. N.Y. Acad. Sci.* 772:152-163; Pisetsky (1996a); Pisetsky (1996b) *Immunity* 5:303-310; Zhao et al. (1996) *Biochem. Pharmacol.* 51:173-182; Yi et al. (1996) *J. Immunol.* 156:558-564; Krieg (1996) *Trends Microbiol.* 4(2):73-76; Krieg et al. (1996) *Antisense Nucleic Acid Drug Dev.* 6:133-139; Klinman et al. (1996); Raz et al. (1996); Sato et al. (1996); Stacey et al. (1996) *J. Immunol.* 157:2116-2122; Ballas et al. (1996); Branda et al. (1996) *J. Lab. Clin. Med.* 128:329-338; Sonehara et al. (1996) *J. Interferon and Cytokine Res.* 16:799-803; Klinman et al. (1997) *J. Immunol.* 158:3635-3639; Sparwasser et al. (1997) *Eur. J. Immunol.* 27:1671-1679; Roman et al. (1997); Carson et al. (1997) *J. Exp. Med.* 186:1621-1622; Chace et al. (1997) *Clin. Immunol. and Immunopathol.* 84:185-193; Chu et al. (1997) *J. Exp. Med.* 186:1623-1631; Lipford et al. (1997a) *Eur. J. Immunol.* 27:2340-2344; Lipford et al. (1997b) *Eur. J. Immunol.* 27:3420-3426; Weiner et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:10833-10837; Macfarlane et al. (1997) *Immunology* 91:586-593; Schwartz et al. (1997) *J. Clin. Invest.* 100:68-73; Stein et al. (1997) *Antisense Technology*, Ch. 11 pp. 241-264, C. Lichtenstein and W. Nellen, Eds., IRL Press; Wooldridge et al. (1997) *Blood* 89:2994-2998; Leclerc et al. (1997) *Cell. Immunol.* 179:97-106; Kline et al. (1997) *J. Invest. Med.* 45(3):282A; Yi et al. (1998a) *J. Immunol.* 160:1240-1245; Yi et al. (1998b) *J. Immunol.* 160:4755-4761; Yi et al. (1998c) *J. Immunol.* 160:4755-4761.

al. (1998c) *J. Immunol.* 160:5898-5906; Yi et al. (1998d) *J. Immunol.* 161:4493-4497; Krieg (1998) *Applied Antisense Oligonucleotide Technology* Ch. 24, pp. 431-448, C.A. Stein and A.M. Krieg, Eds., Wiley-Liss, Inc.; Krieg et al. (1998a) *Trends Microbiol.* 6:23-27; Krieg et al. (1998b) *J. Immunol.* 161:2428-2434; Krieg et al. (1998c) *Proc. Natl. Acad. Sci. USA* 95:12631-12636; Spiegelberg et al. (1998) *Allergy* 53(45S):93-97; Horner et al. (1998) *Cell Immunol.* 190:77-82; Jakob et al. (1998) *J. Immunol.* 161:3042-3049; Redford et al. (1998) *J. Immunol.* 161:3930-3935; Weeratna et al. (1998) *Antisense & Nucleic Acid Drug Development* 8:351-356; McCluskie et al. (1998) *J. Immunol.* 161(9):4463-4466; Gramzinski et al. (1998) *Mol. Med.* 4:109-118; Liu et al. (1998) *Blood* 92:3730-3736; 5 Moldoveanu et al. (1998) *Vaccine* 16: 1216-1224; Brazolot Milan et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:15553-15558; Briode et al. (1998) *J. Immunol.* 161:7054-7062; Briode et al. (1999) *Int. Arch. Allergy Immunol.* 118:453-456; Kovarik et al. (1999) *J. Immunol.* 162:1611-1617; Spiegelberg et al. (1999) *Pediatr. Pulmonol. Suppl.* 18:118-121; Martin- 10 Orozco et al. (1999) *Int. Immunol.* 11:1111-1118; EP 468,520; WO 96/02555; WO 97/28259; WO 98/16247; WO 98/18810; WO 98/37919; WO 98/40100; WO 98/52581; 15 WO 98/52962; WO 98/55495; WO 98/55609 and WO 99/11275. Elkins et al. (1999) *J. Immunol.* 162:2291-2298; WO 98/52962.

20 There remains a serious need to develop effective preventative and therapeutic strategies for HIV infection.

25 All of the cited literature included in the preceding section, as well as the cited literature included in the following disclosure, are hereby incorporated herein by reference.

#### DISCLOSURE OF THE INVENTION

30 The present invention provides immunomodulatory compositions comprising an antigen from human immunodeficiency virus (HIV) and further comprising an immunomodulatory polynucleotide, wherein the immunomodulatory polynucleotide comprises at least one immunostimulatory sequence (ISS). In some embodiments, the immunomodulatory polynucleotide is SEQ ID NO:1 (5'-TGACTGTGAACGTTCGAGATGA-3'). In other embodiments, the immunomodulatory polynucleotide comprises SEQ ID NO:1.

In another embodiment, the immunomodulatory composition comprises an HIV polypeptide or glycoprotein. In some embodiments, the HIV antigen is a gp120 polypeptide.

5 In another embodiment, the immunomodulatory composition comprises the oligonucleotide SEQ ID NO:1 and the HIV gp120 antigen.

10 In some embodiments, the HIV antigen is conjugated to an immunomodulatory polynucleotide. In other embodiments, the HIV antigen is not conjugated to an immunomodulatory polynucleotide and is proximately associated with the immunomodulatory ISS-containing polynucleotide at a distance such that an immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and HIV antigen in solution.

15 The invention also provides for methods of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory polynucleotide comprising an ISS and an HIV antigen. In some embodiments, the immune response modulation comprises the induction of a Th1 response.

20 In some embodiments, the HIV antigen and the oligonucleotide are co-administered. In other embodiments, the HIV antigen is conjugated to an immunomodulatory polynucleotide comprising an ISS. In other embodiments, the HIV antigen is proximately associated with an immunomodulatory polynucleotide comprising an ISS at a distance such that an immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and HIV antigen in solution.

25 Further embodiments include methods of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120 polypeptide and an immunomodulatory polynucleotide, wherein said polynucleotide comprises an immunostimulatory sequence (ISS), wherein a gp120 polypeptide is conjugated to the immunomodulatory polynucleotide, and wherein the immunomodulatory composition is administered to the individual in an amount sufficient to stimulate a specific immune response against gp120. The immune response may comprise production of an anti-gp120 antibody and/or production of T cells.

30 Also included are methods of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120 polypeptide and an immunomodulatory polynucleotide, wherein said

polynucleotide comprises an immunostimulatory sequence (ISS), wherein gp120 polypeptide is not conjugated and is proximately associated to the immunomodulatory polynucleotide at a distance such that an immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and gp120 polypeptide in solution, and wherein the immunomodulatory composition is administered to the individual in an amount sufficient to stimulate a specific immune response against gp120. The immune response may comprise production of an anti-gp120 antibody and/or production of T cells.

Further embodiments include methods of suppressing HIV infection in an individual infected with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection.

In some embodiments, the invention provides methods of delaying development of HIV infection in an individual at risk of infection with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection upon exposure to HIV.

The invention also provides for methods of treating an individual in need of immune modulation comprising administration of an immunomodulatory composition comprising an immunomodulatory oligonucleotide and an antigen, including, but not limited to, individuals suffering from an infectious disease. A further embodiment provides a method for treating individuals infected with human immunodeficiency virus.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the level of IFN- $\gamma$  found in the culture supernatant of splenocytes from mice injected with HIV gp120 compositions.

Figure 2 is a graph depicting serum levels of anti-gp120 IgG2a generated in treated animals.

Figure 3 is a graph depicting serum levels of anti-gp120 IgG1 generated in treated animals.

Figure 4 is a graph depicting anti-gp120 CTL responses from splenocytes of treated animals.

Figure 5 is a graph depicting levels of HIV neutralizing antibodies generated in treated animals.

#### MODES FOR CARRYING OUT THE INVENTION

5 The present invention provides compositions comprising a viral antigen, in particular an HIV antigen, and an immunomodulatory polynucleotide (used interchangeably herein with the term "oligonucleotide"), which, when administered to a host, stimulate an effective antibody and/or cellular response to said antigen. The invention also provides methods of modulating an immune response as well as methods that suppress 10 or ameliorate HIV infection and/or stimulate an HIV-specific immune response which use the compositions described herein.

#### General Techniques

15 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in 20 Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

25

#### Definitions

30 The term "ISS" as used herein refers to oligonucleotide sequences that effect a measurable immune response as measured *in vitro*, *in vivo* and/or *ex vivo*. Examples of measurable immune responses include, but are not limited to, antigen-specific antibody production, secretion of cytokines, activation or expansion of lymphocyte populations such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, B lymphocytes, and the like. Preferably, the ISS sequences preferentially activate a Th1-type response.

As used interchangeably herein, the terms "oligonucleotide" and "polynucleotide" include single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), modified oligonucleotides and oligonucleosides or combinations thereof. The oligonucleotide can be linearly or circularly 5 configured, or the oligonucleotide can contain both linear and circular segments.

Oligonucleotides are polymers of nucleosides joined, generally, through phosphoester linkages. A nucleoside consists of a purine (adenine or guanine or derivative thereof) or pyrimidine (thymine, cytosine or uracil, or derivative thereof) base bonded to a sugar. The four nucleoside units (or bases) in DNA are called deoxyadenosine, deoxyguanosine, 10 deoxythymidine, and deoxycytidine. A nucleotide is a phosphate ester of a nucleoside.

As used herein, the term "conjugate" refers to a complex in which an ISS and an immunomodulatory molecule are linked. Such conjugate linkages include covalent and/or non-covalent linkages.

As used herein, the term "antigen" means a substance that is recognized and bound 15 specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins, polysaccharides, gangliosides and lipids; portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic.

Haptens are included within the scope of "antigen." A hapten is a low molecular weight 20 compound that is not immunogenic by itself but is rendered immunogenic when conjugated with an immunogenic molecule containing antigenic determinants. Antigens suitable for administration with ISS include any molecule capable of eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. A wide variety of molecules are antigens. These include, but are not limited to, sugars, 25 lipids and polypeptides, as well as macromolecules such as complex carbohydrates, and phospholipids. Small molecules may need to be haptenized in order to be rendered antigenic. Preferably, antigens of the present invention include peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *Hemophilus influenza* vaccines, gangliosides and glycoproteins.

As evident by the definition of "antigen", an "HIV antigen" is an antigen derived 30 from a human immunodeficiency virus (HIV). These antigens may thus be, for example, polypeptides and glycoproteins (which may or may not consist of or comprise entire sequences or fragments). For simplicity, the term "HIV antigen" thus includes

polypeptides and glycoproteins, and a term such as "gp120 polypeptide" refers to glycoprotein embodiments, as one skilled in the art readily appreciates that gp120 is a glycoprotein.

5 As used herein, the term "adjuvant" refers to a substance which, when added to an immunogenic agent, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture.

10 As used herein, the term "peptide" includes peptides and proteins that are of sufficient length and composition to effect a biological response, e.g. antibody production or cytokine activity whether or not the peptide is a hapten. Typically, the peptides are of at least six amino acid residues in length. The term "peptide" further includes modified amino acids (whether or not naturally or non-naturally occurring) such modifications including, but not limited to, phosphorylation, glycosylation, pegylation, lipidization and methylation.

15 "Antigenic peptides" can include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-organisms, or fragments of such peptides. An "antigenic peptide" or "antigen polypeptide" accordingly means all or a portion of a polypeptide which exhibits one or more antigenic properties. Thus, a "gp120 antigenic polypeptide" or "gp120 polypeptide antigen" is an amino acid sequence from gp120, whether the entire sequence, a portion of the sequence, 20 and/or a modification of the sequence, which exhibits an antigenic property (i.e., binds specifically to an antibody or a T cell receptor).

25 The term "immunomodulatory" or "modulating an immune response" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, macrophages and the like; increased synthesis of immunostimulatory cytokines including, but not limited to, IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, IFN- $\gamma$ , TNF- $\alpha$  and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses. Examples of immunosuppressive effects include, 30 but are not limited to, a reduction in antigen-specific antibody production such as reduced

IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN- $\gamma$ , which appears to block IL-4 induced class switch to IgE and IgG1, thereby reducing the levels of these antibody subclasses.

5 As used herein, "in conjunction with" refers to administration of one treatment modality in addition to another treatment modality, such as administration of an ISS-containing polynucleotide to a subject in addition to the delivery of, for example, a protease inhibitor (or other treatment for HIV infection and/or AIDS) to the same subject, or 10 administration of two different ISS to the same subject. As such, "in conjunction with" refers to administration of one treatment modality before, during or after delivery of the other treatment modality to the subject.

15 As used herein, and as well-understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial 20 or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

25 As used herein, "delaying" development of HIV infection means to defer, hinder, slow, retard, stabilize, and/or postpone development of the disease(s) associated with HIV infection when compared to not administering the ISS and HIV-antigen containing compositions described herein. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop disease(s) associated with HIV infection. A method that "delays" development of HIV infection is a method that reduces probability of disease development in a given time frame and/or reduces the extent of the disease in a given time frame, when compared to not using the method. Such comparisons are typically based on clinical studies, using a 30 statistically significant number of subjects.

An "individual" or "subject" is a vertebrate, preferably a mammal, and includes but is not limited to domestic animals, farm animals, sports animals, rodents, and primates, including humans.

5      ***Compositions comprising ISS and antigen***

The invention provides compositions and methods useful for stimulating an anti-HIV immune response. A composition of the subject invention (any embodiment of which may be used in the methods described herein) comprises an HIV antigen and an ISS, and said composition is capable of eliciting a desired immune response. In some embodiments, 10 the invention provides compositions comprising ISS and one or more HIV antigenic peptides, such as gp120 polypeptide.

HIV antigens

15      Antigens used in the subject compositions and methods using the compositions are HIV antigens. Such antigens include, but are not limited to, those antigens derived from HIV envelope glycoproteins including, but not limited to, gp160, gp120 and gp41.

Generally, immunomodulatory peptides can be native or synthesized chemically or enzymatically. Any method of chemical synthesis known in the art is suitable. Solution phase peptide synthesis can be used to construct peptides of moderate size or, for the chemical construction of peptides, solid phase synthesis can be employed. Atherton et al. 20 (1981) *Hoppe Seylers Z. Physiol. Chem.* 362:833-839. Proteolytic enzymes can also be utilized to couple amino acids to produce peptides. Kullmann (1987) *Enzymatic Peptide Synthesis*, CRC Press, Inc. Alternatively, the peptide can be obtained by using the biochemical machinery of a cell, or by isolation from a biological source. Recombinant DNA techniques can be employed for the production of peptides. Hames et al. (1987) 25 *Transcription and Translation: A Practical Approach*, IRL Press. Peptides can also be isolated using standard techniques such as affinity chromatography. In certain cases, the peptides are commercially available.

30      Preferably the antigens are peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *H. influenza* vaccines, gangliosides and glycoproteins. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases,

such as for many sterols, fatty acids and phospholipids, the antigenic portions of the molecules are commercially available.

HIV antigens may be obtained using methods known in the art, for example, from native HIV virus extracts, from cells infected with HIV, from purified HIV polypeptides, 5 from recombinantly produced HIV polypeptides and/or as synthetic peptides. For example, purified HIV polypeptides from HIV infected cells are described in U.S. Patent No. 4,725,669. Polynucleotide sequences encoding HIV polypeptides may be cloned into expression vectors as described, for example in U.S. Patent No. 5,817,637, for isolation of polypeptides through the use of recombinant techniques. HIV polypeptides are also 10 commercially available, for example, from Intracel Corporation, Rockville, MD.

To generate HIV antigens, numerous sequences for HIV genes and antigens are known. For example, the Los Alamos National Laboratory HIV Sequence Database collects, curates and annotates HIV nucleotide and amino acid sequences. This database is accessible via the internet, at <http://hiv-web.lanl.gov/>, and in a yearly publication, see 15 *Human Retroviruses and AIDS Compendium* (for example, 1998 edition).

Examples of HIV nucleotide and amino acid sequences from a variety of HIV strains have been described. HIV polypeptides include: gag polypeptides, including membrane anchoring polypeptide p17, core capsid p24 and nucleocapsid proteins p7 and p6; polymerase polypeptides, including protease (p15), reverse transcriptase and RNase H 20 (p66/p51 heterodimer) and integrase (p31); envelope glycoproteins, including gp160, gp120 and gp41; vif polypeptide (p23); vpr polypeptide (p15); vpu polypeptide (p16); nef polypeptide (p27); rev polypeptide (p19) and tat polypeptide (p14). The HIV antigen of the present invention may comprise any HIV polypeptide, or derivative thereof, known in the art including, but not limited to, those described in Ratner et al. (1985) *Nature* 313:277-284; Muesing et al. (1985) *Nature* 313:450-458; Myers et al. (1992) *AIDS Res. Hum. Retroviruses* 8:373-386; McCutchan et al. (1992) *AIDS Res. Hum. Retroviruses* 8:1887-1895; Gurgo et al. (1988) *Virol.* 164:531-536; Berman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5200-5204; Malim et al., 1989, *Cell* 58:205-214.

Amino acid sequences of many HIV peptides and polypeptides, including those of the polymerase polypeptides, are disclosed in U.S. Patent No. 5,843,638. Amino acid 30 sequences of HIV gag polypeptides and peptides, including those for antigens p24 and p17, are described, for example, in U.S. Patent Nos. 5,919,462; 5,859,193; 5,700,469 and

5,612,453. Amino acid sequences of HIV envelope polypeptides, including those for antigens gp41, gp160 and gp120, are described, for example, in U.S. Patent Nos. 5,859,193, 5,338,829 and 5,864,027. Amino acid sequences of HIV tat polypeptides are described, for example, in U.S. Patent No. 5,652,122.

5 In some embodiments, the HIV antigen is a surface glycoprotein, or functional fragment thereof (meaning that the fragment is able to specifically bind to an antibody or a T cell receptor). Accordingly, in some embodiments, the HIV antigen is a gp120 polypeptide (including gp120); a gp41 polypeptide (including gp41); a gp160 polypeptide (including gp160). As is evident to one skilled in the art, synthesis of a composition 10 comprising an ISS-containing polynucleotide and gp120 can lead to production of related compositions comprising other gp120 polypeptides (e.g., fragments and/or modified polypeptides) using well-developed methods known in the art. For example, a fragment (i.e., region or portion) of gp120, such as the CD4<sup>+</sup>-binding region, can be tested for its 15 ability to bind to an anti-gp120 antibody and/or a T cell receptor using standard binding assays. Alternatively, a fragment of gp120 can be tested for its ability to elicit a desired immune response when administered with an ISS-containing polynucleotide.

#### ISS

20 In accordance with the present invention, the immunomodulatory polynucleotide contains at least one ISS, and can contain multiple ISSs. The ISSs can be adjacent within the polynucleotide, or they can be separated by additional nucleotide bases within the 25 polynucleotide.

ISS have been described in the art and may be readily identified using standard assays which indicate various aspects of the immune response, such as cytokine secretion, 30 antibody production, NK cell activation and T cell proliferation. See, e.g., WO 97/28259; WO 98/16247; WO 99/11275; Krieg et al. (1995); Yamamoto et al. (1992); Ballas et al. (1996); Klinman et al. (1997); Sato et al. (1996); Pisetsky (1996a); Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Cowdery et al. (1996) *J. Immunol.* 156:4570-4575; Roman et al. (1997); and Lipford et al. (1997a).

35 The ISS can be of any length greater than 6 bases or base pairs and generally comprises the sequence 5'-cytosine, guanine-3', more particularly comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3' (such as 5'-AACGTT-3'), preferably greater than 15 bases or base pairs, more preferably greater than 20 bases or base pairs in

length. In some embodiments, the ISS comprises the sequence 5'purine, T, C, G, pyrimidine, pyrimidine-3'. In some embodiments, the oligonucleotide of the composition contains at least one octameric ISS. It has been found that a particular set of octanucleotide sequences within oligonucleotide sequences renders the oligonucleotide capable of modulating an immune response. Such oligonucleotide sequences comprise an immunostimulatory octanucleotide sequence (ISS). The ISS of the present invention comprise an octanucleotide sequence which comprises the previously described hexamer and two additional nucleotides 3' of the hexamer. The octameric ISS preferably comprises a CG containing sequence of the general octameric sequence 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine, Cytosine, (Cytosine or Guanine)-3'. Most preferably, the ISS comprises an octamer selected from the group consisting of: AACGTTCC, AACGTTCG, GACGTTCC, and GACGTTCG. Where the immunostimulatory oligonucleotide comprises an RNA sequence, the ISS preferably comprises a single-stranded or double-stranded sequence selected from the group consisting of AACGUUCC, AACGUUCG, GACGUUCC, and GACGUUCG.

Accordingly, an ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'.

In some embodiments, the ISS comprises any of the following sequences:

20 GACGCTCC; GACGTCCC; GACGTTCC; GACGCCCC; AGCGTTCC; AGCGCTCC; AGCGTCCC; AGCGCCCC; AACGTCCC; AACGCCCC; AACGTTCC; AACGCTCC; GGCGTTCC; GGCGCTCC; GGCGTCCC; GGCGCCCC; GACGCTCG; GACGTCCG; GACGCCCG; GACGTTCG; AGCGCTCG; AGCGTTCG; AGCGTCCG; AGCGCCCCG; AACGTCCG; AACGCCCG; AACGTTCG; AACGCTCG; GGCGTTCG; GGCGCTCG; GGCGTCCG; GGCGCCCCG. In some embodiments, the immunomodulatory polynucleotide comprises the sequence 5'-TGACTGTGAACGTTCGAGATGA-3' (SEQ ID NO:1). In other embodiments, the ISS comprises any of the sequences:

25 5'-TGACCGTGAAACGTTCGAGATGA-3';

30 5'-TCATCTCGAACGTTCCACAGTCA-3';

30 5'-TGACTGTGAACGTTCCAGATGA-3';

5'-TCCATAACGTTCGCCTAACGTTCGTC-3';

5'-TGACTGTGAABGTTCCAGATGA-3', where B is 5-bromocytosine;

5'-TGACTGTGAABGTTCGAGATGA-3', where B is 5-bromocytosine and

5'-TGACTGTGAABGTTBGAGATGA-3', where B is 5-bromocytosine.

An ISS and/or ISS-containing polynucleotide may contain modifications.

Modifications of ISS include any known in the art, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. Various such modifications are described below. Multiple bases, sugars, or phosphates in any combination can be substituted in the ISS.

An ISS may be single stranded or double stranded DNA, as well as single or 10 double-stranded RNA or other modified polynucleotides. In general, dsRNA exerts an immunostimulatory effect and is encompassed by the invention.

An ISS may or may not include one or more palindromic regions, which may be present in the hexameric motif described above or may extend beyond the motif. An ISS may comprise additional flanking sequences, some of which are described herein. An ISS 15 may contain naturally-occurring or modified, non-naturally occurring bases, and may contain modified sugar, phosphate, and/or termini. For example, phosphate modifications include, but are not limited to, methyl phosphonate, phosphorothioate, phosphoramidate (bridging or non-bridging), phosphotriester and phosphorodithioate and may be used in any combination. Other non-phosphate linkages may also be used. Preferably, 20 oligonucleotides of the present invention comprise phosphorothioate backbones. Sugar modifications known in the field, such as 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras and others described herein, may also be made and combined with any phosphate modification. Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C- 25 6 of a cytosine of the ISS (e.g., 5-bromocytosine, 5-chlorocytosine, 5-fluorocytosine, 5-iodocytosine). When the same cytosine is methylated, all immunostimulatory activity of the oligonucleotide is lost. Preferably, in such compositions, the cytosine in the third position from the 5' end can be substituted with a cytosine analog, preferably 5- bromocytidine, fluorinated cytosine, or chlorinated cytosine. Some of the modified ISS 30 have approximately the same, if not greater, immunostimulatory activity relative to the same sequence without a modified base.

The ISS-containing oligonucleotide can comprise any other physiologically acceptable modified nucleotide base.

The ISS can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, 5 Ausubel et al. (1987); and Sambrook et al. (1989). When assembled enzymatically, the individual units can be ligated, for example, with a ligase such as T4 DNA or RNA ligase. U.S. Patent No. 5,124,246. Oligonucleotide degradation can be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675.

10 The ISS can also be isolated using conventional polynucleotide isolation procedures. Such procedures include, but are not limited to, hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences, antibody screening of expression libraries to detect shared structural features and synthesis of particular native sequences by the polymerase chain reaction.

15 Circular ISS can be isolated, synthesized through recombinant methods, or chemically synthesized. Where the circular ISS is obtained through isolation or through recombinant methods, the ISS will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using any method described in the literature. See, for instance, Gao et al. (1995) *Nucleic Acids Res.* 23:2025-2029; and Wang 20 et al. (1994) *Nucleic Acids Res.* 22:2326-2333.

25 The techniques for making oligonucleotides and modified oligonucleotides are known in the art. Naturally occurring DNA or RNA, containing phosphodiester linkages, is generally synthesized by sequentially coupling the appropriate nucleoside phosphoramidite to the 5'-hydroxy group of the growing oligonucleotide attached to a solid support at the 3'-end, followed by oxidation of the intermediate phosphite triester to a phosphate triester. Once the desired oligonucleotide sequence has been synthesized, the oligonucleotide is removed from the support, the phosphate triester groups are deprotected to phosphate diesters and the nucleoside bases are deprotected using aqueous ammonia or other bases. See, for example, Beaucage (1993) "Oligodeoxyribonucleotide Synthesis" in *Protocols for 30 Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, Totowa, NJ; Warner et al. (1984) *DNA* 3:401 and U.S. Patent No. 4,458,066.

The ISS can also contain phosphate-modified oligonucleotides. Synthesis of polynucleotides containing modified phosphate linkages or non-phosphate linkages is also known in the art. For a review, see Matteucci (1997) "Oligonucleotide Analogs: an Overview" in *Oligonucleotides as Therapeutic Agents*, (D.J. Chadwick and G. Cardew, ed.) 5 John Wiley and Sons, New York, NY. The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the oligonucleotides of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphonate, phosphorothioate, phosphorodithioate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified 10 nucleotides and oligonucleotides, *per se*, is also known and need not be described here in detail. Peyrottes et al. (1996) *Nucleic Acids Res.* 24:1841-1848; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz et al. (1996) *Nucleic Acids Res.* 24:2966- 15 2973. For example, synthesis of phosphorothioate oligonucleotides is similar to that described above for naturally occurring oligonucleotides except that the oxidation step is replaced by a sulfurization step (Zon (1993) "Oligonucleoside Phosphorothioates" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) 20 Humana Press, pp. 165-190). Similarly the synthesis of other phosphate analogs, such as phosphotriester (Miller et al. (1971) *JACS* 93:6657-6665), non-bridging phosphoramidates (Jager et al. (1988) *Biochem.* 27:7247-7246), N3' to P5' phosphoramidates (Nelson et al. 25 (1997) *JOC* 62:7278-7287) and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used (Stirchak et al. (1989) *Nucleic Acids Res.* 17:6129-6141). Oligonucleotides with phosphorothioate backbones can be more immunogenic than those with phosphodiester 30 backbones and appear to be more resistant to degradation after injection into the host. Braun et al. (1988) *J. Immunol.* 141:2084-2089; and Latimer et al. (1995) *Mol. Immunol.* 32:1057-1064.

The oligonucleotide of the invention can comprise ribonucleotides (containing 35 ribose as the only or principal sugar component), deoxyribonucleotides (containing deoxyribose as the principal sugar component), or, as is known in the art, modified sugars or sugar analogs can be incorporated in the ISS. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar can

be in pyranosyl or in a furanosyl form. In the ISS, the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-0-alkylribose, and the sugar can be attached to the respective heterocyclic bases either in  $\alpha$  or  $\beta$  anomeric configuration. Sugar modifications include, but are not limited to, 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) *per se* is known, and need not be described here, except to the extent such preparation can pertain to any specific example. Sugar modifications may also be made and combined with any phosphate modification in the preparation of an ISS.

10 The heterocyclic bases, or nucleic acid bases, which are incorporated in the ISS can be the naturally-occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally-occurring and synthetic modifications of said principal bases.

15 Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the present invention are satisfied, the ISS can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, 20 the heterocyclic base in the ISS includes, but is not limited to, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the ISS via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the 25 pyrazolopyrimidines via the 1-position.

25 The ISS may comprise at least one modified base. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." Examples of 30 base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS. Preferably, the electron-withdrawing moiety is a halogen. Such modified cytosines can include, but are not limited to,

azacytosine, 5-bromocytosine, bromouracil, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, 5-fluorocytosine, fluoropyrimidine, fluorouracil, 5,6-dihydrocytosine, 5-iodocytosine, hydroxyurea, iodouracil, 5-nitrocytosine, uracil, and any other pyrimidine analog or modified pyrimidine.

5 The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, 10 present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also been described (including, but not limited to, e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

15 In some embodiments, an ISS-containing polynucleotide is less than about any of the following lengths (in bases or base pairs): 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; 10. In some embodiments, an ISS-containing polynucleotide is greater than about any of the following lengths (in bases or base pairs): 8; 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500; 10000; 20000; 50000.

20 ISS-HIV antigen

25 The ISS-containing polynucleotide-HIV antigen compositions described herein may be in a number of configurations. Generally, the ISS-containing polynucleotide may be in an admixture (i.e., in solution) or proximately associated to each other by any of a number of means as described below. In some embodiments, the HIV antigen, such as gp120 polypeptide, is conjugated to the ISS-containing polynucleotide. In some embodiments, the HIV antigen is proximately associated with the ISS-containing polynucleotide (and is not conjugated). As described below, spatial proximation (proximate association) can be accomplished in a number of ways, including encapsidation, via affixation to a platform or adsorption onto a surface. Generally, and most preferably, an ISS-containing 30 polynucleotide and HIV antigen are proximately associated at a distance effective to enhance the immune response generated compared to the administration of the ISS and HIV antigen as an admixture. In some embodiments, HIV antigen and ISS-containing

polynucleotide are in an admixture (i.e., in solution). If in an admixture (i.e., solution) the ISS and antigen are maintained at concentrations effective to modulate an immune response.

Accordingly, the invention provides compositions (and methods using these compositions) in which the HIV antigen(s) is conjugated to an ISS-containing polynucleotide. Preferably, the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. Preferably, the ISS comprises or consists of SEQ ID NO:1. The HIV antigen may be any of the antigens described above, and preferably is a gp120 polypeptide (which includes gp120).

10 The ISS portion can be coupled with the antigen portion of a conjugate in a variety of ways, including covalent and/or non-covalent interactions.

15 The link between the portions can be made at the 3' or 5' end of the ISS, or at a suitably modified base at an internal position in the ISS. If the antigen is a peptide and contains a suitable reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N<sup>4</sup> amino group of cytosine residues. Depending on the number and location of cytosine residues in the ISS, specific coupling at one or more residues can be achieved.

20 Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the antigen of interest.

25 Where the antigen is a peptide, this portion of the conjugate can be attached to the 3'-end of the ISS through solid support chemistry. For example, the ISS portion can be added to a polypeptide portion that has been pre-synthesized on a support. Haralambidis et al. (1990a) *Nucleic Acids Res.* 18:493-499; and Haralambidis et al. (1990b) *Nucleic Acids Res.* 18:501-505. Alternatively, the ISS can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the ISS from the support, a terminal thiol group is left at the 3'-end of the oligonucleotide (Zuckermann et al. (1987) *Nucleic Acids Res.* 15:5305-5321; and Corey et al. (1987) *Science* 238:1401-1403) or a terminal amino group is left at the 3'-end of the oligonucleotide (Nelson et al. (1989) *Nucleic Acids Res.* 17:1781-1794). Conjugation of the amino-modified ISS to amino groups of the peptide can be performed as described in

Benoit et al. (1987) *Neuromethods* 6:43-72. Conjugation of the thiol-modified ISS to carboxyl groups of the peptide can be performed as described in Sinah et al. (1991) *Oligonucleotide Analogues: A Practical Approach*, IRL Press. Coupling of an oligonucleotide carrying an appended maleimide to the thiol side chain of a cysteine residue of a peptide has also been described. Tung et al. (1991) *Bioconjug. Chem.* 2:464-465.

The peptide portion of the conjugate can be attached to the 5'-end of the ISS through an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis. Preferably, while the oligonucleotide is fixed to the 10 solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl. Agrawal et al. (1986) *Nucleic Acids Res.* 14:6227-6245; Connolly (1985) *Nucleic Acids Res.* 13:4485-4502; Kremsky et al. (1987) *Nucleic Acids Res.* 15:2891-2909; Connolly (1987) *Nucleic Acids Res.* 15:3131-3139; Bischoff et al. (1987) *Anal. Biochem.* 164:336-344; Blanks et al. 15 (1988) *Nucleic Acids Res.* 16:10283-10299; and U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802. Subsequent to deprotection, the amine, thiol, and carboxyl functionalities can be used to covalently attach the oligonucleotide to a peptide. Benoit et al. (1987); and Sinah et al. (1991).

An ISS-antigen conjugate can also be formed through non-covalent interactions, 20 such as ionic bonds, hydrophobic interactions, hydrogen bonds and/or van der Waals attractions.

Non-covalently linked conjugates can include a non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an ISS. Roget et al. (1989) *Nucleic Acids Res.* 17:7643-7651. Incorporation of a 25 streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated oligonucleotide.

Non-covalent associations can also occur through ionic interactions involving an ISS and residues within the antigen, such as charged amino acids, or through the use of a linker portion comprising charged residues that can interact with both the oligonucleotide 30 and the antigen. For example, non-covalent conjugation can occur between a generally negatively-charged ISS and positively-charged amino acid residues of a peptide, e.g., polylysine, polyarginine and polyhistidine residues.

Non-covalent conjugation between ISS and antigens can occur through DNA binding motifs of molecules that interact with DNA as their natural ligands. For example, such DNA binding motifs can be found in transcription factors and anti-DNA antibodies.

5 The linkage of the ISS to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al. (1988) *Nucleic Acids Symp. Ser.* 19:189-192), oligonucleotide-fatty acid conjugates (Grabarek et al. (1990) *Anal. Biochem.* 185:131-135; and Staros et al. (1986) *Anal. Biochem.* 156:220-222), and oligonucleotide-sterol conjugates. Boujrad et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5728-5731.

10 The linkage of the oligonucleotide to an oligosaccharide can be formed using standard known methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin. O'Shannessy et al. (1985) *J. Applied Biochem.* 7:347-355.

15 The linkage of a circular ISS to a peptide or antigen can be formed in several ways. Where the circular ISS is synthesized using recombinant or chemical methods, a modified nucleoside is suitable. Ruth (1991) in *Oligonucleotides and Analogues: A Practical Approach*, IRL Press. Standard linking technology can then be used to connect the circular ISS to the antigen or other peptide. Goodchild (1990) *Bioconjug. Chem.* 1:165. Where the circular ISS is isolated, or synthesized using recombinant or chemical methods, the linkage 20 can be formed by chemically activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or other peptide.

25 Additional methods for the attachment of peptides and other molecules to oligonucleotides can be found in U.S. Patent No. 5,391,723; Kessler (1992) "Nonradioactive labeling methods for nucleic acids" in Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press; and Geoghegan et al. (1992) *Bioconjug. Chem.* 3:138-146.

30 The peptide portion can be attached to a modified cytosine or uracil at any position in the ISS. The incorporation of a "linker arm" possessing a latent reactive functionality, such as an amine or carboxyl group, at C-5 of the modified base provides a handle for the peptide linkage. Ruth, *4th Annual Congress for Recombinant DNA Research*, p. 123.

The invention also provides for compositions which comprise an ISS-antigen conjugate or an ISS-antigen admixture and an adjuvant where, upon co-administration, the

association of ISS-antigen and adjuvant is effective to enhance an immune response compared to the co-administration of the ISS-antigen without adjuvant. In such compositions, the adjuvant is maintained in association with ISS-antigen so as to recruit and activate target cells to the ISS-antigen.

5 The invention further provides for compositions in which ISS and an antigen are in proximate association at a distance effective to enhance the immune response generated compared to the administration of the ISS and the antigen as an admixture (i.e., compared to the co-administration of the ISS and antigen in solution). In some embodiments, the proximate association is by means other than conjugation. Preferably, the ISS and antigen are proximately associated at a distance of about 0.04  $\mu$ m to about 100  $\mu$ m, more preferably, at a distance of about 0.1  $\mu$ m to about 20  $\mu$ m, even more preferably, at a distance of about 0.15  $\mu$ m to about 10  $\mu$ m. Targets of the ISS-antigen conjugate or the ISS-antigen admixture include, but are not limited to, antigen presenting cells (APCs), such as macrophages, dendritic cells, and/or lymphocytes, lymphatic structures, such as lymph nodes and/or the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found, such as skin, lungs, and/or gastrointestinal tract.

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Enhancement of an immune response by a composition in which an ISS and an immunomodulatory agent are proximately associated refers to a modulation of an immune response following administration of said composition as compared to the immune response following administration of the ISS and immunomodulatory agent freely soluble with respect to each other. Enhancement of an immune response includes modulation of an immune response including, but not limited to, stimulation, suppression and a shift in the type of immune response, for instance, between a Th1-type response and a Th2-type response.

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25 An ISS may be proximately associated with an antigen(s) in a number ways. In some embodiments, an ISS and antigen are proximately associated by encapsulation. In other embodiments, an ISS and antigen are proximately associated by linkage to a platform molecule. A "platform molecule" (also termed "platform") is a molecule containing sites which allow for attachment of the ISS and antigen(s). In other embodiments, an ISS and antigen are proximately associated by adsorption onto a surface, preferably a carrier 30 particle.

5 In some embodiments, the methods of the invention employ an encapsulating agent that can maintain the proximate association of the ISS and HIV antigen until the complex is available to the target (or compositions comprising such encapsulating agents). Preferably, the composition comprising ISS, antigen and encapsulating agent is in the form of adjuvant oil-in-water emulsions, microparticles and/or liposomes. More preferably, adjuvant oil-in-water emulsions, microparticles and/or liposomes encapsulating an ISS-immunomodulatory molecule are in the form of particles from about 0.04  $\mu$ m to about 100  $\mu$ m in size, preferably any of the following ranges: from about 0.1  $\mu$ m to about 20  $\mu$ m; from about 10 0.15  $\mu$ m to about 10  $\mu$ m; from about 0.05  $\mu$ m to about 1.00  $\mu$ m; from about 0.05  $\mu$ m to about 0.5  $\mu$ m.

Colloidal dispersion systems, such as microspheres, beads, macromolecular complexes, nanocapsules and lipid-based system, such as oil-in-water emulsions, micelles, mixed micelles and liposomes can provide effective encapsulation of ISS-containing compositions.

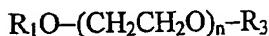
15 The encapsulation composition further comprises any of a wide variety of components. These include, but are not limited to, alum, lipids, phospholipids, lipid membrane structures (LMS), polyethylene glycol (PEG) and other polymers, such as polypeptides, glycopeptides, and polysaccharides.

20 Polypeptides suitable for encapsulation components include any known in the art and include, but are not limited to, fatty acid binding proteins. Modified polypeptides contain any of a variety of modifications, including, but not limited to glycosylation, phosphorylation, myristylation, sulfation and hydroxylation. As used herein, a suitable 25 polypeptide is one that will protect an ISS-containing composition to preserve the immunomodulatory activity thereof. Examples of binding proteins include, but are not limited to, albumins such as bovine serum albumin (BSA) and pea albumin.

Other suitable polymers can be any known in the art of pharmaceuticals and 30 include, but are not limited to, naturally-occurring polymers such as dextrans, hydroxyethyl starch, and polysaccharides, and synthetic polymers. Examples of naturally occurring polymers include proteins, glycopeptides, polysaccharides, dextran and lipids. The additional polymer can be a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as PEG, polyoxyethylated polyols (POP), such as polyoxyethylated glycerol

(POG), polytrimethylene glycol (PTG) polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone (PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, co-polymers, or block co-polymers of two or more different synthetic monomers.

5 PEGs constitute a diverse group of molecules. A general formula for PEGs is as follows:



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where  $R_1$  and  $R_3$  are independently H,  $H_3C$ , OH, or a linear or branched, substituted or unsubstituted alkyl group and n is an integer between 1 and about 1,000. The term "PEG" includes both unsubstituted ( $R_1$  and  $R_3$  = H) as well as substituted PEG. The PEGs for use in encapsulation compositions of the present invention are either purchased from chemical suppliers or synthesized using techniques known to those of skill in the art.

15 The term "LMS", as used herein, means lamellar lipid particles wherein polar head groups of a polar lipid are arranged to face an aqueous phase of an interface to form membrane structures. Examples of the LMSs include liposomes, micelles, cochleates (i.e., generally cylindrical liposomes), microemulsions, unilamellar vesicles, multilamellar vesicles, and the like.

20 A preferred colloidal dispersion system of this invention is a liposome. In mice immunized with a liposome-encapsulated antigen, liposomes appeared to enhance a Th1-type immune response to the antigen. Aramaki et al. (1995) *Vaccine* 13:1809-1814. As used herein, a "liposome" or "lipid vesicle" is a small vesicle bounded by at least one and possibly more than one bilayer lipid membrane. Liposomes are made artificially from phospholipids, glycolipids, lipids, steroids such as cholesterol, related molecules, or a combination thereof by any technique known in the art, including but not limited to sonication, extrusion, or removal of detergent from lipid-detergent complexes. A liposome can also optionally comprise additional components, such as a tissue targeting component. 25 It is understood that a "lipid membrane" or "lipid bilayer" need not consist exclusively of lipids, but can additionally contain any suitable other components, including, but not limited to, cholesterol and other steroids, lipid-soluble chemicals, proteins of any length,

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and other amphipathic molecules, providing the general structure of the membrane is a sheet of two hydrophilic surfaces sandwiching a hydrophobic core. For a general discussion of membrane structure, see *The Encyclopedia of Molecular Biology* by J. Kendrew (1994). For suitable lipids see e.g., Lasic (1993) "Liposomes: from Physics to Applications" Elsevier, Amsterdam.

5 Preferably, a liposomal composition is chosen that allows the membrane to be formed with reproducible qualities, such as diameter, and is stable in the presence of elements expected to occur where the liposome is to be used, such as physiological buffers and circulating molecules. Preferably, the liposome is resilient to the effects of 10 manipulation by storage, freezing, and mixing with pharmaceutical excipients.

Lipids suitable for incorporation into lipid membrane structures include, but are not limited to, natural, semi-synthetic or synthetic mono- or di-glycerophospholipids including, but not limited to, phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), 15 phosphatidylglycerols (PGs), phosphatidylinositols (PIs), phosphatidic acids (PAs), phosphatidylserines (PSs), glycero- and cardiolipins. Sphingolipids such as sphingomyelin (SM) and cerebrosides can also be incorporated. While natural phospholipids occur with the phospho moiety at the *sn*-3 position and hydrophobic chains at the *sn*-1 and *sn*-2 20 positions, synthetic lipids can have alternative stereochemistry with, e.g., the phospho group at the *sn*-1 or *sn*-2 positions. Furthermore, the hydrophobic chains can be attached to the glycerol backbone by acyl, ether, alkyl or other linkages. Derivatives of these lipids are 25 also suitable for incorporation into liposomes. Derivatives suitable for use include, but are not limited to, haloalkyl derivatives, including those in which all or some of the hydrogen atoms of the alkyl chains are substituted with, e.g., fluorine. In addition, cholesterol and other amphipathic steroids, bolaamphiphiles (lipids with polar moieties at either end of the molecule which form monolayer membranes) and polyglycerolmonoalkylthers can also be incorporated. Liposomes can be composed of a single lipid or mixtures of two or more 30 different lipids.

In one embodiment, the lipid bilayer of the liposome is formed primarily from phospholipids. Preferably, the phospholipid composition is a complex mixture, comprising a combination of PS and additional lipids such as PC, PA, PE, PG and SM, PI, and/or 35 cardiolipin (diphosphatidylglycerol). If desired, SM can be replaced with a greater proportion of PC, PE, or a combination thereof. PS can be optionally replaced with PG.

The composition is chosen so as to confer upon the LMS both stability during storage and administration.

Practitioners of ordinary skill will readily appreciate that each phospholipid in the foregoing list can vary in its structure depending on the fatty acid moieties that are esterified to the glycerol moiety of the phospholipid. Generally, most commercially available forms of a particular phospholipid can be used. However, phospholipids containing particular fatty acid moieties may be preferred for certain applications.

Processes for preparing liposomes containing ISS-containing compositions are known in the art. The lipid vesicles can be prepared by any suitable technique known in the art. Methods include, but are not limited to, microencapsulation, microfluidization, LLC method, ethanol injection, freon injection, the "bubble" method, detergent dialysis, hydration, sonication, and reverse-phase evaporation. Reviewed in Watwe et al. (1995) *Curr. Sci.* 68:715-724. Techniques may be combined in order to provide vesicles with the most desirable attributes.

A general process for preparing liposomes containing ISS and antigen-containing compositions is as follows. An aqueous dispersion of liposomes is prepared from membrane components, such as phospholipids (e.g. PS, PC, PG, SM and PE) and glycolipids according to any known methods. See, e.g., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980). The liposomes can further contain sterols, dialkylphosphates, diacylphosphatidic acids, stearylamine,  $\alpha$ -tocopherol, etc., in the liposomal membrane.

To the liposomal dispersion thus prepared is added an aqueous solution of the ISS and antigen-containing composition and the mixture is allowed to stand for a given period of time, preferably under warming at a temperature above the phase transition temperature of the membrane or above 40°C, followed by cooling to thereby prepare liposomes containing the ISS and antigen-containing composition in the liposomal membrane.

Alternatively, the desired liposomes can also be prepared by previously mixing the above-described membrane components and ISS and antigen-containing composition and treating the mixture in accordance with known methods for preparing liposomes.

Optionally, the LMS also includes steroids to improve the rigidity of the membrane. Any amount of a steroid can be used. Suitable steroids include, but are not limited to, cholesterol and cholestanol. Other molecules that can be used to increase the rigidity of the membrane include, but are not limited to, cross-linked phospholipids.

Other preferred LMSs for use *in vivo* are those with an enhanced ability to evade the reticuloendothelial system, which normally phagocytoses and destroys non-native materials, thereby giving the liposomes a longer period in which to reach the target cell. Effective lipid compositions in this regard are those with a large proportion of SM and cholesterol, or SM and PI. LMSs with prolonged circulation time also include those that comprise the monosialoganglioside GM1, glucuronide, or PEG.

The invention encompasses use of LMSs containing tissue or cellular targeting components. Such targeting components are components of a LMS that enhance its accumulation at certain tissue or cellular sites in preference to other tissue or cellular sites when administered to an intact animal, organ, or cell culture. A targeting component is generally accessible from outside the liposome, and is therefore preferably either bound to the outer surface or inserted into the outer lipid bilayer. A targeting component can be *inter alia* a peptide, a region of a larger peptide, an antibody specific for a cell surface molecule or marker, or antigen binding fragment thereof, a nucleic acid, a carbohydrate, a region of a complex carbohydrate, a special lipid, or a small molecule such as a drug, hormone, or hapten, attached to any of the aforementioned molecules. Antibodies with specificity toward cell type-specific cell surface markers are known in the art and are readily prepared by methods known in the art.

The LMSs can be targeted to any cell type toward which a therapeutic treatment is to be directed, e.g., a cell type which can modulate and/or participate in an immune response. Such target cells and organs include, but are not limited to, APCs, such as macrophages, dendritic cells and lymphocytes, lymphatic structures, such as lymph nodes and the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found.

The LMS compositions of the present invention can additionally comprise surfactants. Surfactants can be cationic, anionic, amphiphilic, or nonionic. A preferred class of surfactants are nonionic surfactants; particularly preferred are those that are water soluble. Nonionic, water soluble surfactants include polyoxyethylene derivatives of fatty alcohols, fatty acid ester of fatty alcohols and glyceryl esters, wherein the polyoxyethylene group is coupled via an ether linkage to an alcohol group. Examples include, but are not limited to, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil

derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyoxyethylene fatty acid ester and polyoxyethylene alkyl ethers.

The LMS compositions encompassed herein include micelles. The term "micelles" as used herein means aggregates which form from tenside molecules in aqueous solutions above a specific temperature (Krafft point) or a characteristic concentration, the critical micellization concentration (cmc). When the cmc is exceeded, the monomer concentration remains practically constant and the excess tenside molecules form micelles. Micelles are thermodynamically stable association colloids of surfactant substances in which the hydrophobic radicals of the monomers lie in the interior of the aggregates and are held together by hydrophobic interaction; the hydrophilic groups face the water and by solvation provide the solubility of the colloid. Micelles occur in various shapes (spheres, rods, discs) depending on the chemical constitution of the tenside and on the temperature, concentration or ionic strength of the solution. Reaching the cmc is manifest by abrupt changes in surface tension, osmotic pressure, electrical conductivity and viscosity.

A process for preparing micelles containing ISS and antigen compositions is as follows. A micelle-forming surfactant, such as polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyoxyethylene fatty acid ester, and polyoxyethylene alkyl ethers, alkyl glycosides, is added to water at a concentration above the cmc to prepare a micellar dispersion. To the micellar dispersion is added an aqueous solution of an ISS and antigen-containing composition and the mixture is allowed to stand for a given period of time, preferably under warming at 40°C or higher, followed by cooling, to thereby prepare micelles containing ISS and antigen-containing compositions in the micellar membrane. Alternatively, the desired micelles can also be prepared by previously mixing the above-described micelle-forming substances and ISS and antigen-containing compositions and treating the mixture according to known methods for micelle formation.

In embodiments in which an ISS and antigen are proximately associated by linkage to a platform molecule, the platform may be proteinaceous or non-proteinaceous (i.e., organic). Examples of proteinaceous platforms include, but are not limited to, albumin, gammaglobulin, immunoglobulin (IgG) and ovalbumin. Borel et al. (1990) *Immunol. Methods* 126:159-168; Dumas et al. (1995) *Arch. Dermatol. Res.* 287:123-128; Borel et al.

(1995) *Int. Arch. Allergy Immunol.* 107:264-267; Borel et al. (1996) *Ann. N.Y. Acad. Sci.* 778:80-87. A platform is multi-valent (i.e., contains more than one binding, or linking, site) to accommodate binding to both an ISS and antigen. Other examples of polymeric platforms are dextran, polyacrylamide, ficoll, carboxymethylcellulose, polyvinyl alcohol, and poly D-glutamic acid/D-lysine.

5 The principles of using platform molecules are well understood in the art. Generally, a platform contains, or is derivatized to contain, appropriate binding sites for ISS and antigen. In addition, or alternatively, ISS and/or antigen is derivatized to provide appropriate linkage groups. For example, a simple platform is a bi-functional linker (i.e., 10 has two binding sites), such as a peptide. Further examples are discussed below.

15 Platform molecules may be biologically stabilized, i.e., they exhibit an *in vivo* excretion half-life often of hours to days to months to confer therapeutic efficacy, and are preferably composed of a synthetic single chain of defined composition. They generally have a molecular weight in the range of about 200 to about 200,000, preferably about 200 to about 50,000 (or less, such as 30,000). Examples of valency platform molecules are 20 polymers (or are comprised of polymers) such as polyethylene glycol (PEG; preferably having a molecular weight of about 200 to about 8000), poly-D-lysine, polyvinyl alcohol, polyvinylpyrrolidone, D-glutamic acid and D-lysine (in a ratio of 3:2). Other molecules that may be used are albumin and IgG.

20 Other platform molecules suitable for use within the present invention are the chemically-defined, non-polymeric valency platform molecules disclosed in U.S. patent 5,552,391. Other homogeneous chemically-defined valency platform molecules suitable for use within the present invention are derivatized 2,2'-ethylenedioxydiethylamine (EDDA) and triethylene glycol (TEG).

25 Additional suitable valency platform molecules include, but are not limited to, tetraaminobenzene, heptaaminobetacyclodextrin, tetraaminopentaerythritol, 1,4,8,11-tetraazacyclotetradecane (Cyclam) and 1,4,7,10-tetraazacyclododecane (Cyclen).

30 In general, these platforms are made by standard chemical synthesis techniques. PEG must be derivatized and made multivalent, which is accomplished using standard techniques. Some substances suitable for conjugate synthesis, such as PEG, albumin, and IgG are available commercially.

Conjugation of an ISS and antigen to a platform molecule may be effected in any number of ways, typically involving one or more crosslinking agents and functional groups on the antigen and ISS platform and platform molecule. Platforms and ISS and antigen must have appropriate linking groups. Linking groups are added to platforms using standard synthetic chemistry techniques. Linking groups may be added to polypeptide antigens and ISS using either standard solid phase synthetic techniques or recombinant techniques. Recombinant approaches may require post-translational modification in order to attach a linker, and such methods are known in the art.

As an example, polypeptides contain amino acid side chain moieties containing functional groups such as amino, carboxyl or sulphydryl groups that serve as sites for coupling the polypeptide to the platform. Residues that have such functional groups may be added to the polypeptide if the polypeptide does not already contain these groups. Such residues may be incorporated by solid phase synthesis techniques or recombinant techniques, both of which are well known in the peptide synthesis arts. When the polypeptide has a carbohydrate side chain(s) (or if the antigen is a carbohydrate), functional amino, sulphydryl and/or aldehyde groups may be incorporated therein by conventional chemistry. For instance, primary amino groups may be incorporated by reaction with ethylenediamine in the presence of sodium cyanoborohydride, sulphydryls may be introduced by reaction of cysteamine dihydrochloride followed by reduction with a standard disulfide reducing agent, while aldehyde groups may be generated following periodate oxidation. In a similar fashion, the platform molecule may also be derivatized to contain functional groups if it does not already possess appropriate functional groups.

Hydrophilic linkers of variable lengths are useful for connecting ISS and antigen to platform molecules. Suitable linkers include linear oligomers or polymers of ethylene glycol. Such linkers include linkers with the formula  $R^1S(CH_2CH_2O)_nCH_2CH_2O(CH_2)_mCO_2R^2$  wherein  $n = 0-200$ ,  $m = 1$  or  $2$ ,  $R^1 = H$  or a protecting group such as trityl,  $R^2 = H$  or alkyl or aryl, e.g., 4-nitrophenyl ester. These linkers are useful in connecting a molecule containing a thiol reactive group such as haloacetyl, maleiamide, etc., via a thioether to a second molecule which contains an amino group via an amide bond. These linkers are flexible with regard to the order of attachment, i.e., the thioether can be formed first or last.

In embodiments in which an ISS and antigen are proximately associated by adsorption onto a surface, the surface may be in the form of a carrier particle (for example, a nanoparticle) made with either an inorganic or organic core. Examples of such nanoparticles include, but are not limited to, nanocrystalline particles, nanoparticles made by the polymerization of alkylcyanoacrylates and nanoparticles made by the polymerization of methylidene malonate. Additional surfaces to which an ISS and antigen may be adsorbed include, but are not limited to, activated carbon particles and protein-ceramic nanoplates.

Adsorption of polynucleotides and polypeptides to a surface for the purpose of delivery of the adsorbed molecules to cells is well known in the art. See, for example, Douglas et al. (1987) *Crit. Rev. Ther. Drug. Carrier Syst.* 3:233-261; Hagiwara et al. (1987) *In Vivo* 1:241-252; Bousquet et al. (1999) *Pharm. Res.* 16:141-147; and Kossovsky et al., U.S. Patent 5,460,831. Preferably, the material comprising the adsorbent surface is biodegradable. Adsorption of an ISS and/or antigen to a surface may occur through non-covalent interactions, including ionic and/or hydrophobic interactions.

In general, characteristics of nanoparticles, such as surface charge, particle size and molecular weight, depend upon polymerization conditions, monomer concentration and the presence of stabilizers during the polymerization process (Douglas et al., 1987). The surface of carrier particles may be modified, for example, with a surface coating, to allow or enhance adsorption of the ISS and/or antigen. Carrier particles with adsorbed ISS and/or antigen may be further coated with other substances. The addition of such other substances may, for example, prolong the half-life of the particles once administered to the subject and/or may target the particles to a specific cell type or tissue, as described herein.

Nanocrystalline surfaces to which an ISS and antigen may be adsorbed have been described (see, for example, U.S. Patent 5,460,831). Nanocrystalline core particles (with diameters of 1  $\mu\text{m}$  or less) are coated with a surface energy modifying layer that promotes adsorption of polypeptides, polynucleotides and/or other pharmaceutical agents. As described in U.S. Patent 5,460,831, for example, a core particle is coated with a surface that promotes adsorption of an oligonucleotide and is subsequently coated with an antigen preparation, for example, in the form of a lipid-antigen mixture. Such nanoparticles are self-assembling complexes of nanometer sized particles, typically on the order of 0.1  $\mu\text{m}$ , that carry an inner layer of ISS and an outer layer of antigen.

Another adsorbent surface are nanoparticles made by the polymerization of alkylcyanoacrylates. Alkylcyanoacrylates can be polymerized in acidified aqueous media by a process of anionic polymerization. Depending on the polymerization conditions, the small particles tend to have sizes in the range of 20 to 3000 nm, and it is possible to make nanoparticles specific surface characteristics and with specific surface charges (Douglas et al., 1987). For example, oligonucleotides may be adsorbed to polyisobutyl- and polyisohexylcyanoacrylate nanoparticles in the presence of hydrophobic cations such as tetraphenylphosphonium chloride or quaternary ammonium salts, such as cetyltrimethyl ammonium bromide. Oligonucleotide adsorption on these nanoparticles appears to be mediated by the formation of ion pairs between negatively charged phosphate groups of the nucleic acid chain and the hydrophobic cations. See, for example, Lambert et al. (1998) *Biochimie* 80:969-976, Chavany et al. (1994) *Pharm. Res.* 11:1370-1378; Chavany et al. (1992) *Pharm. Res.* 9:441-449. Polypeptides may also be adsorbed to polyalkylcyanoacrylate nanoparticles. See, for example, Douglas et al., 1987; Schroeder et al. (1998) *Peptides* 19:777-780.

Another adsorbent surface are nanoparticles made by the polymerization of methylidene malonate. For example, as described in Bousquet et al., 1999, polypeptides adsorbed to poly(methylidene malonate 2.1.2) nanoparticles appear to do so initially through electrostatic forces followed by stabilization through hydrophobic forces.

In some embodiments, the invention provides compositions comprising ISS, HIV antigen, and further comprising an adjuvant (including, but not limited to, alum, lipid emulsions, and polylactide/polyglycolide microparticles). In another embodiment, the invention provides compositions comprising ISS, an HIV antigen and an adjuvant whereby the ISS/antigen/adjuvant are co-administered. In other embodiments, the HIV antigen and ISS are conjugated or proximately associated. Preferably, the immunogenic composition contains an amount of an adjuvant sufficient to potentiate the immune response to the immunogen. Preferably, adjuvants include, but are not limited to, oil-in-water emulsions, water-in oil emulsions, alum (aluminum salts), liposomes and microparticles, including but not limited to, polystyrene, starch, polyphosphazene and polylactide/polyglycosides. More preferably, the ISS and antigen are co-administered with alum. More preferably, the ISS and antigen are co-administered with liposomes. Still more preferably, the ISS and antigen are co-administered with an oil-in-water emulsion. In some embodiments, the ISS and

antigen can be associated with an adjuvant through covalent and/or non-covalent interactions. An example of such non-covalent interactions includes, but is not limited to, adsorption of the ISS and antigen to microparticles described herein.

Suitable adjuvants also include, but are not limited to, squalene mixtures (SAF-1),  
5 muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875, as well as, lipid-based adjuvants and others described herein. For veterinary use and  
10 for production of antibodies in animals, mitogenic components of Freund's adjuvant (both complete and incomplete) can be used.

*Methods of modulating immune responses with ISS and antigen*

The present invention also provides methods for the use of any of the compositions described herein (generally, ISS in conjunction with an HIV antigen) in stimulation of an immune response, preferably an HIV-antigen specific immune response. Preferably, as used in such methods, the ISS provides an adjuvant-like activity in the generation of a Th1-type immune response to the antigen. The invention also provides methods of treating an individual in need of immune modulation comprising administration of an  
15 immunomodulatory composition comprising an immunomodulatory oligonucleotide and an HIV antigen, including, but not limited to, individuals infected with HIV or at risk of being infected with HIV. As the term "treatment" indicates to one skilled in the art, treating individuals as described herein can encompass any of the following: alleviation of one or more symptoms associated with HIV infection; diminishment of extent of HIV infection  
20 (including any disease that would arise due to HIV infection); stabilized condition; delay or slowing of progression; amelioration or palliation of any disease state associated with HIV infection (including AIDS); suppressing HIV infection.

Accordingly, the invention provides methods in which administration of ISS-containing polypeptide and HIV antigen described herein is used to modulate an antigen-specific immune response in an individual, in particular an HIV-specific response. In some embodiments, the modulation of the immune response is by induction or increase of a Th1 response. In some embodiments, the invention provides methods of preventing and/or  
30

delaying development of HIV infection. In some embodiments, the invention provides methods of ameliorating or palliating HIV infection, including any symptom of or associated with HIV infection. In some embodiments, the invention provides methods of suppressing HIV infection. In some embodiments, the invention provides methods of stimulating a specific immune response against an HIV antigen, preferably against gp120. 5 All such methods may be particularly beneficial to individuals infected with, or at risk of infection with, HIV.

In some embodiments, the invention provides methods of stimulating a specific immune response against an HIV antigen (which have been listed above) comprising 10 administering an ISS-containing polynucleotide and the HIV antigen in an amount sufficient to stimulate an immune response against the administered HIV antigen. Preferably, the HIV antigen is a gp120 polypeptide (including gp120). Accordingly, the invention provides methods of stimulating a specific immune response against gp120 in an individual comprising administering an ISS-containing polynucleotide and a gp120 15 polypeptide in an amount sufficient to stimulate an anti-gp120 immune response (i.e., a gp120-specific immune response). The immune response may comprise production of anti-gp120 antibody and/or production of gp120-specific T cells, especially gp120-specific cytotoxic T cells (CTLs). The immune response may also comprise stimulation of Th1 cells (including production of cytokines associated with stimulation of Th1 cells). Example 20 1 illustrates stimulating both a gp120 specific antibody response and a gp120-specific CTL response, as well as a Th1 mediated response, based on administration to a mammal (mouse) a conjugate of gp120 and SEQ ID NO:1.

For these embodiments, the ISS-containing polynucleotide and HIV antigen may be administered as an admixture (i.e., not proximately associated) or proximately associated. 25 Preferably, the ISS-containing polynucleotide and HIV antigen (such as gp120) are administered proximately associated such that the immune response is enhanced as compared to administering ISS-containing polynucleotide and HIV antigen as an admixture. In some embodiments, ISS-containing polynucleotide and gp120 are proximately associated, which includes any of the above-described embodiments for 30 proximate association, including conjugation. In some embodiments, the ISS-containing polynucleotide is SEQ ID NO:1, although any of the ISS described herein may be used.

Preferably, the immune response stimulated according to the invention is biased toward the Th1-type phenotype and away from the Th2-type phenotype. With reference to the invention, stimulating a Th1-type immune response can be determined *in vitro* or *ex vivo* by measuring cytokine production from cells treated with ISS as compared to those treated without ISS. Methods to determine the cytokine production of cells include those methods described herein and any known in the art. The type of cytokines produced in response to ISS treatment indicate a Th1-type or a Th2-type biased immune response by the cells. As used herein, the term "Th1-type biased" cytokine production refers to the measurable increased production of cytokines associated with a Th1-type immune response 5 in the presence of a stimulator as compared to production of such cytokines in the absence of stimulation. Examples of such Th1-type biased cytokines include, but are not limited to, IL-2, IL-12, and IFN- $\gamma$ . In contrast, "Th2-type biased cytokines" refers to those associated with a Th2-type immune response, and include, but are not limited to, IL-4, IL-5, IL-10 and IL-13. Cells useful for the determination of ISS activity include cells of the immune 10 system, primary cells isolated from a host and/or cell lines, preferably APCs and lymphocytes, even more preferably macrophages and T cells.

15

Stimulating a Th1-type immune response can also be measured in a host treated with an ISS-antigen composition and can be determined by any method known in the art including, but not limited to: (1) a reduction in levels of IL-4 measured before and after 20 antigen-challenge; or detection of lower (or even absent) levels of IL-4 in an ISS-antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS; (2) an increase in levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) in an 25 ISS-antigen treated host as compared to an antigen-primed or, primed and challenged, control treated without ISS; (3) IgG2a antibody production in an ISS-antigen treated host as compared to a control treated without ISS; and/or (4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even 30 absent) levels of antigen-specific IgE in an ISS-antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS. A variety of these determinations can be made by measuring cytokines made by APCs and/or lymphocytes, preferably macrophages and/or T cells, *in vitro* or *ex vivo* using methods described herein

or any known in the art. Methods to determine antibody production include any known in the art.

The Th1-type biased cytokine induction which occurs as a result of ISS administration produces enhanced cellular immune responses, such as those performed by 5 NK cells, cytotoxic killer cells, Th1 helper and memory cells. These responses are particularly beneficial for use in protective or therapeutic vaccination against viruses, fungi, protozoan parasites, bacteria, allergic diseases and asthma, as well as tumors.

For all of the methods described herein, an effective amount of ISS-containing 10 polynucleotide and HIV antigen is administered, that is, an amount sufficient to achieve the desired result(s), depending on the objectives of treatment. An effective amount may be given in single or divided doses (i.e., in one or more administrations). Generally, if the ISS-containing polynucleotide and HIV antigen are administered in proximate association to each other (such as in a conjugate form), a dosage range of the ISS-antigen composition may be, for example, from about any of the following: 1 to 500 µg, 100 to 400 µg, 200 to 15 300 µg, 1 to 100 µg, 100 to 200 µg, 300 to 400 µg, 400 to 500 µg. In these compositions, the molar ratio of ISS-containing polynucleotide to antigen may vary. If the ISS-containing polynucleotide and HIV antigen are not proximately associated, i.e., administered as an admixture, generally the dosage ranges are higher, such as, for the ISS-containing 20 polynucleotide, for example, from about any of the following: 10 to 10,000 µg, 2000 to 8000 µg, 4000 to 6000 µg, 10 to 500 µg, 500 to 1000 µg, 1000 to 2000 µg, 2000 to 3000 µg, 6000 to 7000 µg, 7000 to 8000 µg, 8000 to 9000 µg, 9000 to 10,000 µg; and for the 25 HIV antigen, for example, from about any of the following: 0.1 to 500 µg, 1.0 to 100 µg, 5 to 50 µg, 0.1 to 1.0 µg, 1.0 to 10 µg, 50 to 200 µg, 200 to 400 µg, 300 to 500 µg.

The effectiveness of ISS/HIV antigen administration can be monitored by several 30 criteria. Assays for cytokines (including Th1-associated cytokines) have been described above and herein, and are known in the art. Other assays for an immune response are also known in the art, such as assays for antibodies (such as ELISA) and T cells. As is well known in the art, the treated subject may also be monitored for clinical features which accompany the HIV infection. For example, subjects may be monitored for presence and/or levels of p24 protein (which indicates amount of virus present and can also indicate degree of neutralization of virus); and reduction in a symptom associated with infection (such as incidence of opportunistic infections). Individuals may also be monitored for levels of T helper

populations, wherein an increase indicates an effective amount has been administered. For example, after treatment, the individual may be assessed for improvement in a number of clinical parameters including, but not limited to, reduction of incidence and/or symptoms of opportunistic infections. An opportunistic infection, as is well known in the art, may be any infection (whether viral or bacterial) that arise from immunosuppression due to HIV infection. For methods in which HIV infection is suppressed, any indicator of HIV infection, such as presence of HIV RNA, HIV DNA, T cell count, presence of neutralizing anti-HIV antibodies (as measured by p24 levels) or HIV antigen, or symptom associated with HIV infection may be checked and/or monitored using methods known in the art. For methods in which a symptom associated with HIV infection is ameliorated, any such symptom may be checked and/or monitored to confirm that an effective amount of ISS-containing polynucleotide and HIV antigen have been administered. For methods in which HIV infection is palliated or treated, any indicia of HIV infection (such as those listed above) may be checked and/or monitored that an effective amount has been administered.

The selection of a particular composition, dosage regimen (*i.e.*, dose, timing and repetition) and route of administration will depend on a number of different factors, including, but not limited to, the subject's medical history and features of the condition and the subject being treated. The assessment of such features and the design of an appropriate therapeutic regimen is ultimately the responsibility of the prescribing physician. The particular dosage regimen may be determined empirically.

*Individuals receiving ISS and HIV antigen*

Individuals suitable for receiving ISS-containing polynucleotide and antigen include those who have been infected by HIV as well as those who are at risk of being infected by and/or exposed to HIV. Infection by HIV is indicated by any suitable assay or measurable parameter, such as detection of HIV DNA in a clinical (*i.e.*, biological) sample; detection of number of T helper cells; detection of an HIV antigen or anti-HIV antibody; one or more symptoms associated with HIV infection, such as any symptom associated with an opportunistic infection. Such detection systems and clinical parameters are known in the art.

Another type of individual suitable for receiving ISS-containing polynucleotide and HIV antigen are those individuals at risk for infection with HIV. These risk groups, known

in the art, include, for example, individuals who engage in so-called "unprotected" sexual relations; individuals who are intravenous drug users; and health care workers.

*Administration and assessment*

ISS may be administered with HIV antigen in a number of ways. ISS-containing 5 polynucleotide(s) and HIV antigen may be administered as an admixture (i.e., in solution) or, more preferably, be administered spatially proximate with respect to each other, whether by conjugation or by other means of effecting spatial proximation, which have been described above.

The ISS-containing polynucleotide may be any of those described above. 10 Preferably, the ISS-containing polynucleotide administered comprises the formula 5' purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'; more preferably, 5'-A, A, C, G, T, T, C, G-3'. Another preferred embodiment uses SEQ ID NO:1.

The ISS and the HIV antigen(s) can be administered together in the form of a 15 conjugate or co-administered in an admixture sufficiently close in time so as to modulate an immune response. Preferably, the ISS and antigen are administered simultaneously. The term "co-administration" as used herein refers to the administration of at least two different substances sufficiently close in time to modulate an immune response. Preferably, co-administration refers to simultaneous administration of at least two different substances.

The ISS and antigen can be administered as an ISS-antigen conjugate and/or they 20 can be co-administered as a complex in the form of an admixture, such as in an emulsion, and/or administered in spatial proximation such that the immune response is enhanced as compared to administering in a solution. As described above, the association of the ISS and the antigen molecules in an ISS-antigen conjugate can be through covalent interactions and/or through non-covalent interactions, including high affinity and/or low affinity 25 interactions. Examples of non-covalent interactions that can couple an ISS and an antigen in an ISS-antigen conjugate include, but are not limited to, ionic bonds, hydrophobic interactions, hydrogen bonds and van der Waals attractions.

In another embodiment, ISS and antigen can be administered in conjunction with 30 one or more immunomodulatory facilitator. Thus, the invention provides compositions comprising ISS and an immunomodulatory facilitator. As used herein, the term "immunomodulatory facilitator" refers to molecules which support and/or enhance the immunomodulatory activity of an ISS and/or an antigen. Examples of immunomodulatory

facilitators can include co-stimulatory molecules, such as cytokines, and/or adjuvants. The ISS, antigen and facilitator can be administered as an ISS-antigen-facilitator conjugate and/or they can be co-administered as a complex in the form of an admixture, such as in an emulsion. The association of the ISS, the antigen and the facilitator molecules in an ISS-  
5 antigen-facilitator conjugate can be through covalent interactions and/or through non-covalent interactions, including high affinity and/or low affinity interactions. Examples of non-covalent interactions that can couple an ISS, an antigen and a facilitator in an ISS-antigen-facilitator conjugate include, but are not limited to, ionic bonds, hydrophobic interactions, hydrogen bonds and van der Waals attractions.

10 Immunomodulatory facilitators include, but are not limited to, co-stimulatory molecules (such as cytokines, chemokines, targeting protein ligand, trans-activating factors, peptides, and peptides comprising a modified amino acid) and adjuvants (such as alum, lipid emulsions, and polylactide/polyglycolide microparticles).

15 Among suitable immunomodulatory cytokine peptides for administration with ISS and antigen are the interleukins (e.g., IL-1, IL-2, IL-3, etc.), interferons (e.g., IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ ), erythropoietin, colony stimulating factors (e.g., G-CSF, M-CSF, GM-CSF) and TNF- $\alpha$ . Preferably, immunostimulatory peptides for use in conjunction with ISS and antigen are those that stimulate Th1-type immune responses, such as IL-12 (Bliss et al.  
20 (1996) *J. Immunol.* 156:887-894), IL-18, TNF- $\alpha$ ,  $\beta$  and  $\gamma$ , and/or transforming growth factor (TGF)- $\alpha$ .

25 Peptides administered with ISS and antigen can also include amino acid sequences that mediate protein binding to a specific receptor or that mediate targeting to a specific cell type or tissue. Examples include, but are not limited to, antibodies or antibody fragments, peptide hormones such as human growth hormone, and enzymes. Immunomodulatory peptides also include peptide hormones, peptide neurotransmitters and peptide growth factors. Co-stimulatory molecules such as B7 (CD80), trans-activating proteins such as transcription factors, chemokines such as macrophage chemotactic protein (MCP) and other chemoattractant or chemotactic peptides are also useful peptides for administration with ISS and antigen.

30 The invention also provides for the administration of ISS and antigen in conjunction with an adjuvant. Administration of an antigen with an ISS and an adjuvant leads to a potentiation of a immune response to the antigen and thus, can result in an enhanced

immune response compared to that which results from a composition comprising the ISS and antigen alone. For example, we have shown that administration of an antigen with an ISS and an adjuvant leads to an enhanced primary immune response. Examples of suitable adjuvants have been discussed above.

5 As with all immunogenic compositions, the immunologically effective amounts of the components must be determined empirically. Factors to be considered include the antigenicity, whether or not ISS and/or antigen will be complexed with or covalently attached to an immunomodulatory facilitator, an adjuvant or carrier protein or other carrier, route of administration and the number of immunizing doses to be administered. Such 10 factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The compositions of the present invention can be administered alone or in combination with other pharmaceutical and/or immunogenic and/or immunostimulatory agents and can be combined with a physiologically acceptable carrier thereof. The 15 effective amount and method of administration of the particular ISS and antigen formulation can vary based on the individual patient and the stage of the disease and other factors evident to one skilled in the art. The route(s) of administration useful in a particular application are apparent to one of skill in the art. Routes of administration include but are not limited to topical, dermal, transdermal, transmucosal, epidermal, parenteral, 20 gastrointestinal, and naso-pharyngeal and pulmonary, including transbronchial and transalveolar. A suitable dosage range is one that provides sufficient ISS-containing composition to attain a tissue concentration of about 1-10  $\mu$ M as measured by blood levels. The absolute amount given to each patient depends on pharmacological properties such as 25 bioavailability, clearance rate and route of administration.

25 As described herein, APCs and tissues with high concentration of APCs are preferred targets for the ISS and antigen-containing compositions. Thus, administration of ISS and antigen-containing compositions to mammalian skin and/or mucosa, where APCs are present in relatively high concentration, is preferred.

30 The present invention provides ISS and antigen-containing compositions suitable for topical application including, but not limited to, physiologically acceptable implants, ointments, creams, rinses and gels. Topical administration is, for instance, by a dressing or bandage having dispersed therein a delivery system, or by direct administration of a

delivery system into incisions or open wounds. Creams, rinses, gels or ointments having dispersed therein an ISS and antigen-containing composition are suitable for use as topical ointments or wound filling agents.

Preferred routes of dermal administration are those which are least invasive.

5 Preferred among these means are transdermal transmission, epidermal administration and subcutaneous injection. Of these means, epidermal administration is preferred for the greater concentrations of APCs expected to be in intradermal tissue.

10 Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the ISS and antigen-containing composition to penetrate the skin and enter the blood stream. Compositions suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference.

15 For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission can be accomplished using commercially available patches which deliver their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for 20 contemporaneous use of an absorption promoter.

25 An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

30 For transdermal transmission, low-frequency ultrasonic delivery is also a suitable method. Mitragotri et al. (1995) *Science* 269:850-853. Application of low-frequency ultrasonic frequencies (about 1 MHz) allows the general controlled delivery of therapeutic compositions, including those of high molecular weight.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APCs to the site of irritation.

5 An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tines which can be used to irritate the skin and attract APCs to the site of irritation, to take up ISS and antigen-containing compositions transferred from the end of the tines. For example, the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France contains a device suitable for introduction of ISS and antigen-containing compositions.

10 The device (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, PA) consists of a plastic container having a syringe plunger at one end and a tine disk at the other. The tine disk supports a multiplicity of narrow diameter tines of a length which will just scratch the outermost layer of epidermal cells. Each of the tines in the MONO-VACC kit is coated with old tuberculin; in the present invention, each needle is 15 coated with a pharmaceutical composition of ISS and antigen-containing composition. Use of the device is preferably according to the manufacturer's written instructions included with the device product. Similar devices which can also be used in this embodiment are those which are currently used to perform allergy tests.

20 Another suitable approach to epidermal administration of ISS and antigen-containing compositions is by use of a chemical which irritates the outermost cells of the epidermis, thus provoking a sufficient immune response to attract APCs to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR. 25 This approach can also be used to achieve epithelial administration in the mucosa. The chemical irritant can also be applied in conjunction with the mechanical irritant (as, for example, would occur if the MONO-VACC type tine were also coated with the chemical irritant). The ISS and antigen-containing compositions can be suspended in a carrier which also contains the chemical irritant or coadministered therewith.

30 Another delivery method for administering ISS and antigen-containing compositions makes use of non-lipid polymers, such as a synthetic polycationic amino polymer. Leff (1997) *Bioworld* 86:1-2.

Parenteral routes of administration include, but are not limited to, electrical (iontophoresis) or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Compositions suitable for parenteral administration include, but are not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for injection of the ISS and antigen-containing compositions.

Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. The invention includes ISS and antigen-containing compositions suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable, powders, pills or liquids for ingestion and suppositories for rectal administration.

In some embodiments, the ISS-containing polynucleotide and HIV antigen are administered via mucosal immunization (i.e., administration to the mucosa). Mucosal immunization may be by any of a number of means, such as by topical application to the mucosa (including by spray or drops) or injection into the mucosa, in addition to those described below.

Naso-pharyngeal and pulmonary routes of administration include, but are not limited to, by-inhalation, transbronchial and transalveolar routes. The invention includes ISS and antigen-containing compositions suitable for by-inhalation administration including, but not limited to, various types of aerosols for inhalation, as well as powder forms for delivery systems. Devices suitable for by-inhalation administration of ISS and antigen-containing compositions include, but are not limited to, atomizers and vaporizers. Atomizers and vaporizers filled with the powders are among a variety of devices suitable for use in by-inhalation delivery of powders. See, e.g., Lindberg (1993) Summary of Lecture at Management Forum 6-7 December 1993 "Creating the Future for Portable Inhalers."

The methods of producing suitable devices for injection, topical application, atomizers and vaporizers are known in the art and will not be described in detail.

The choice of delivery routes can be used to modulate the immune response elicited. For example, IgG titers and CTL activities were identical when an influenza virus vector was administered via intramuscular or epidermal (gene gun) routes; however, the muscular inoculation yielded primarily IgG2a, while the epidermal route yielded mostly

IgG1. Pertmer et al. (1996) *J. Virol.* 70:6119-6125. Thus, one of skill in the art can take advantage of slight differences in immunogenicity elicited by different routes of administering the immunomodulatory compositions of the present invention.

Analysis (both qualitative and quantitative) of the immune response to ISS and antigen-containing compositions can be by any method known in the art, including, but not limited to, measuring antigen-specific antibody production, activation of specific populations of lymphocytes such as CD4<sup>+</sup> T cells or NK cells, and/or production of cytokines such as IFN, IL-2, IL-4, or IL-12. Methods for measuring specific antibody responses include enzyme-linked immunosorbent assay (ELISA) and are well known in the art. Measurement of numbers of specific types of lymphocytes such as CD4<sup>+</sup> T cells can be achieved, for example, with fluorescence-activated cell sorting (FACS). Cytotoxicity assays are known in the art and can be performed, for instance, as described in Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523. Serum concentrations of cytokines can be measured, for example, by ELISA. These and other assays to evaluate the immune response to an immunogen are well known in the art. See, for example, *Selected Methods in Cellular Immunology* (1980) Mishell and Shiigi, eds., W.H. Freeman and Co.

The above-mentioned compositions and methods of administration are meant to describe but not limit the methods of administering the ISS and antigen-containing compositions of the invention. The methods of producing the various compositions and devices are within the ability of one skilled in the art and are not described in detail here.

The following examples are provided to illustrate, but not limit, the invention.

#### EXAMPLES

25

#### EXAMPLE 1

##### Selective Induction of a Th1-type Response in a Host after Administration of a Composition Comprising an ISS and antigen

As described herein, a Th1-type immune response is associated with the production of specific cytokines, such as IFN- $\gamma$ , and results in production of CTLs.

To determine if a Th1-type immune response would be produced in mice receiving ISS and antigen compositions according to the invention, mice were immunized with HIV

gp120 protein, with and without co-administration of ISS oligonucleotides. The ISS and gp120 were administered in the form of an ISS-gp120 conjugate or as an admixture in saline. A set of mice received gp120 without ISS oligonucleotide.

*Conjugation of oligonucleotide with HIV gp120*

5        Phosphorothioate ISS oligonucleotides were coupled to thiol groups on the protein by the following technique. Recombinant gp120, expressed in CHO cells, was obtained from Quality Biologicals, Inc. Five hundred micrograms of gp120 (4.2 nmole) was treated with 20-50 fold excess of sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate) at room temperature for 2 hours. The maleimido-modified 10      gp120 was purified from unreacted sulfo-SMCC by filtration through a NAP-25 column. A 5'-disulfide modified ISS oligonucleotide (Glen Research, 5'-Amino Modifier 6) was reduced to the thiol by treatment with tricarboxyethyl phosphine (TCEP) in PBS. Following 15      purification by gel filtration and concentration by diafiltration, 10-30 molar excess of the thiol-activated oligonucleotide was incubated with the maleimido-modified gp120 at room 15      temperature over night. Free oligonucleotide was removed and the oligonucleotide-gp120 conjugate was concentrated using spin columns.

15      The conjugate was analyzed, after electrophoresis on a 4-20% SDS-PAGE gel, by UV-shadowing, CB-staining and immunoblotting with an anti-gp120 antibody. All of these analyses demonstrated successful conjugation of the protein and the oligonucleotide. 20      Quantitation of the oligonucleotide/protein molar ratio was determined by a UV assay comparing the conjugate to standards composed of gp120 and molar ratios of the oligonucleotide.

25      The ISS oligonucleotide used in the conjugate was prepared by Trilink BioTechnologies (San Diego, CA) and has the sequence 5'-TGACTGTGAAACGTTCGAGATGA-3' [SEQ ID NO:1].

*Immunization and Immune Response*

30      Generally, for these experiments, groups of four, eight week old female BALB/c mice were immunized once intradermally with 10 µg ISS-gp120 conjugate, 10 µg ISS mixed with gp120 in saline, or 10 µg gp120. Mice were bled every two weeks post-immunization, serum separated and stored at -20°C for later analysis. Three days prior to sacrifice, mice were injected intravenously with 5 µg gp120. Naïve mice are also included in the experiments.

The cytokine secretion profile, antigen-dependent antibody responses and antigen-dependent CTL responses of these mice were tested *in vitro*.

Cytokine secretion was determined by ELISA tests. Generally, to measure cytokine secretion, splenocytes were harvested and resuspended in RP10 medium at  $5 \times 10^5$  5 splenocytes per well in 96 well flat-bottomed tissue culture microtiter plates. Culture medium alone as control or gp120 protein was added to triplicate wells. Culture supernatants were sampled at 48 and 72 hours and then analyzed by ELISA for cytokine levels. The results of such an experiment are depicted in Figure 1. Cells from mice injected with the ISS-gp120 conjugate secreted IFN- $\gamma$ , a cytokine associated with a Th1-type immune response. Cells from mice injected with gp120 mixed with ISS in saline and cells from mice injected with gp120 alone did not secrete detectable levels of IFN- $\gamma$ .  
10

Anti-gp120 antibodies of the IgG2a and IgG1 isotypes were measured by ELISA as described in Raz et al. (1996) and Sato et al. (1996). IgG2a and IgG1 results from such an experiment are depicted in Figure 2 and 3, respectively. As shown in Figure 2, 15 administration of gp120 alone or in a mixture with ISS resulted in almost no anti-gp120 IgG2a production, whereas administration of an ISS-gp120 conjugate generated a significant level of anti-gp120 IgG2a antibody. As described herein, the IgG2a antibody isotype is indicative of a Th1-type immune response.

gp120-specific CTL responses were determined as described in Sato et al. (1996).  
20 As depicted in Figure 4, the cells from mice that received ISS-gp120 conjugate had very strong anti-gp120 CTL activity. Cells from mice injected with gp120 mixed with ISS in saline and cells from mice injected with gp120 alone had very little CTL activity.

Serum from the injected mice were also tested for the presence of HIV neutralizing 25 antibodies by the determination of HIV p24 levels. Results of such an experiment are depicted in Figure 5.

CLAIMS

We claim:

1. An immunomodulatory composition comprising an human immunodeficiency virus (HIV) antigen and further comprising an immunomodulatory polynucleotide, wherein said polynucleotide comprises an immunostimulatory sequence (ISS).  
5
2. The immunomodulatory composition of claim 1, wherein the immunomodulatory polynucleotide comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, 10 G-3'.  
10
3. The immunomodulatory composition of claim 1, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.  
15
4. The immunomodulatory composition of claim 1, wherein the antigen is an HIV gp120 polypeptide.  
15
5. An immunomodulatory composition according to claim 4, wherein the immunomodulatory polynucleotide is SEQ ID NO:1 and wherein the antigen is HIV gp120.  
20
6. The immunomodulatory composition of claim 1, wherein the HIV antigen is conjugated to the immunomodulatory polynucleotide.  
20
7. The immunomodulatory composition of claim 6, wherein the HIV antigen is a gp120 polypeptide.  
25
8. The immunomodulatory composition of claim 7, wherein the HIV antigen is gp120 and the immunomodulatory polynucleotide comprises SEQ ID NO:1.  
25
9. The immunomodulatory composition of claim 1, wherein the HIV antigen is not conjugated to the immunomodulatory polynucleotide and is proximately associated to the immunomodulatory polynucleotide and at a distance such that an immune response is  
30

enhanced compared to co-administration of the immunomodulatory polynucleotide and antigen in solution.

10. The immunomodulatory composition of claim 9, wherein the HIV antigen is a gp120 polypeptide.
- 5
11. The immunomodulatory composition of claim 10, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.
- 10
12. A method of modulating an immune response comprising administration of an immunomodulatory composition according to claim 1 in an amount sufficient to modulate the immune response.
- 15
13. The method of claim 12, wherein the modulating of an immune response comprises induction of a Th1 response.
14. The method of claim 12, wherein the immunomodulatory polynucleotide and HIV antigen are co-administered.
- 20
15. The method of claim 12, wherein the immunomodulatory polynucleotide and HIV antigen are conjugated.
16. The method of claim 12, wherein the immunomodulatory polynucleotide and HIV antigen are not conjugated and are proximately associated at a distance such that an immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and antigen in solution.
- 25
17. The method of claim 15, wherein the HIV antigen is a gp120 polypeptide.
- 30
18. The method of claim 17, wherein the HIV antigen is gp120.

19. The method of claim 18, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

20. The method of claim 16, wherein the HIV antigen is a gp120 polypeptide.

5

21. The method of claim 20, wherein wherein the HIV antigen is gp120.

22. The method of claim 21, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

10

23. The method of claim 17, wherein the modulating of an immune response comprises induction of a Th1 response.

15

24. The method of claim 20, wherein the modulating of an immune response comprises induction of a Th1 response.

20

25. A method of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120 polypeptide and an immunomodulatory polynucleotide, wherein said polynucleotide comprises an immunostimulatory sequence (ISS), wherein the gp120 polypeptide is conjugated to the immunomodulatory polynucleotide, and wherein the immunomodulatory composition is administered to the individual in an amount sufficient to stimulate a specific immune response against gp120.

25

26. The method of claim 25, wherein the polypeptide is gp120.

27. The method of claim 26, wherein gp120 is conjugated to the immunomodulatory polynucleotide.

30

28. The method of claim 27, wherein the immunomodulatory polynucleotide comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

29. The method of claim 28, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

30. The method of claim 25, wherein the specific immune response comprises production  
5 of anti-gp120 antibody.

31. The method of claim 25, wherein the specific immune response comprises production  
of gp120-specific T cells.

10 32. The method of claim 31, wherein the gp120-specific T cells are cytotoxic T cells.

33. A method of stimulating a specific immune response against gp120 in an individual,  
comprising administering an immunomodulatory composition comprising a gp120  
15 polypeptide and an immunomodulatory polynucleotide, wherein said polynucleotide  
comprises an immunostimulatory sequence (ISS), wherein the gp120 polypeptide is not  
conjugated and is proximately associated to the immunomodulatory polynucleotide at a  
distance such that an immune response is enhanced compared to co-administration of the  
immunomodulatory polynucleotide and gp120 polypeptide in solution, and wherein the  
immunomodulatory composition is administered to the individual in an amount sufficient  
20 to stimulate a specific immune response against gp120.

34. The method of claim 33, wherein the polypeptide is gp120.

35. The method of claim 34, wherein gp120 is conjugated to the immunomodulatory  
25 polynucleotide.

36. The method of claim 35, wherein the immunomodulatory polynucleotide comprises the  
sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

30 37. The method of claim 36, wherein the immunomodulatory polynucleotide comprises  
SEQ ID NO:1.

38. The method of claim 33, wherein the specific immune response comprises production of anti-gp120 antibody.
39. The method of claim 33, wherein the specific immune response comprises production of gp120-specific T cells.  
5
40. The method of claim 29, wherein the gp120-specific T cells are cytotoxic T cells.
41. A method of suppressing HIV infection in an individual infected with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection.  
10
42. A method of delaying development of HIV infection in an individual at risk of infection with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection upon exposure to HIV.  
15
43. A method of treating an individual in need of immune modulation, wherein the individual has been infected with HIV, comprising administration of the immunomodulatory composition of claim 1 to the individual.  
20

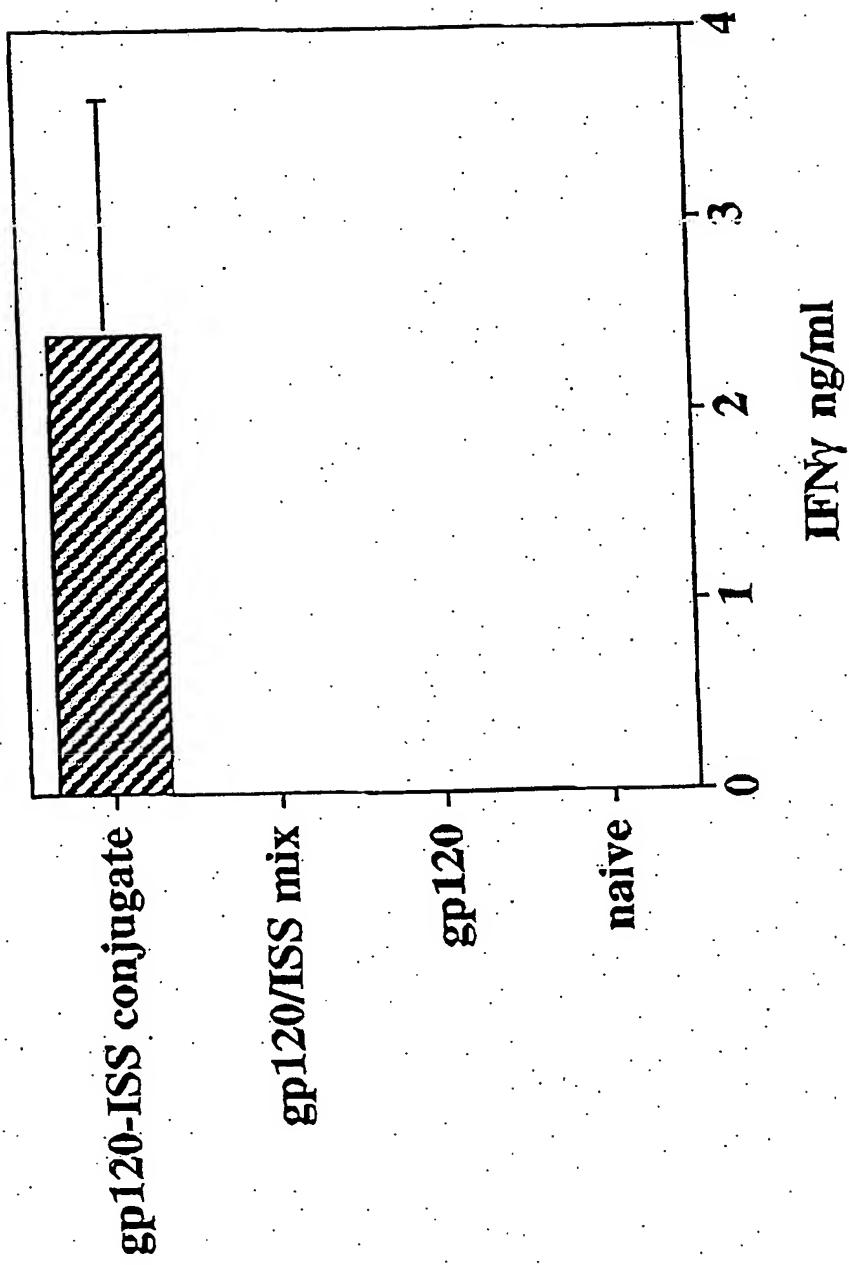
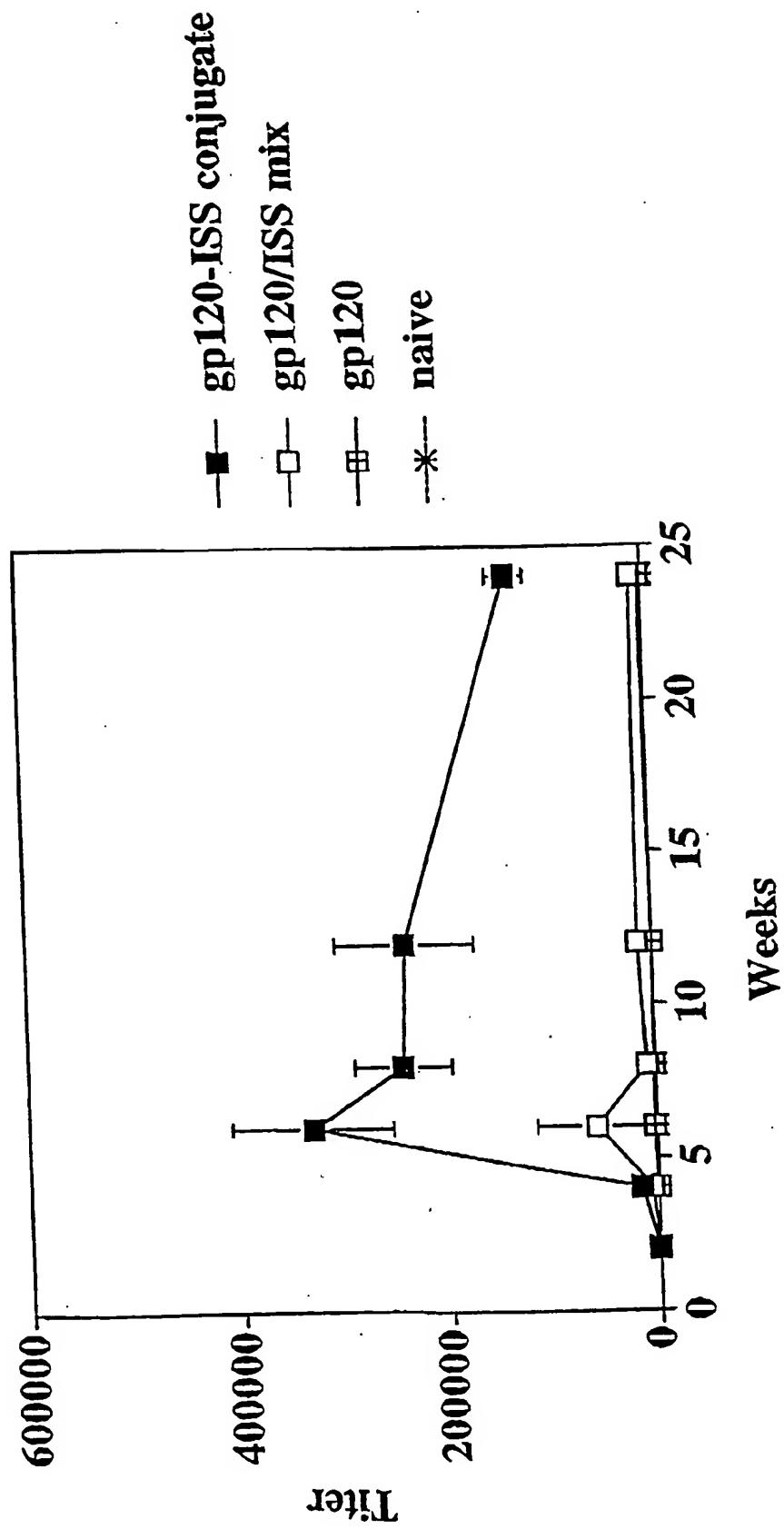


Figure 1



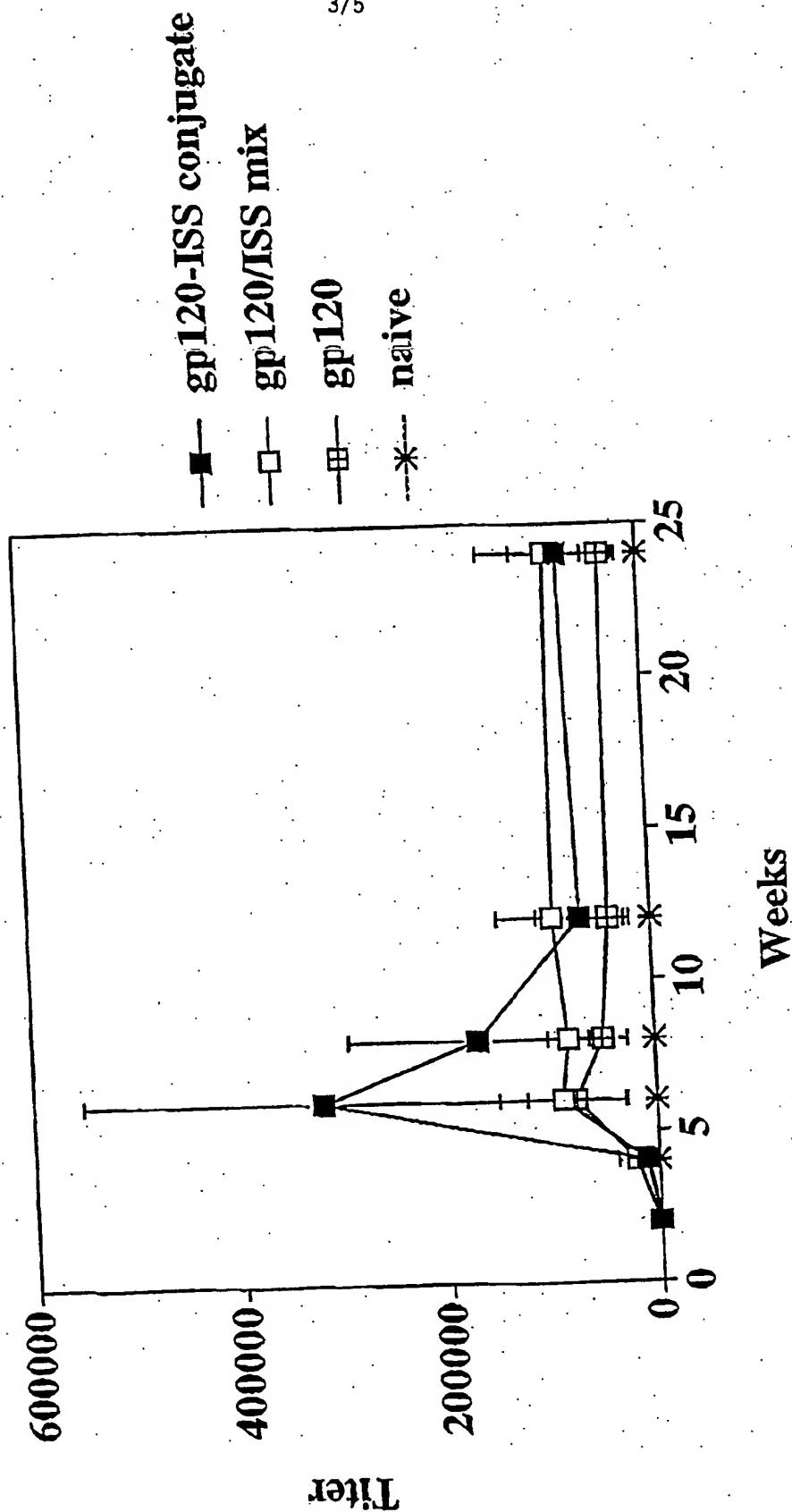


Figure 3

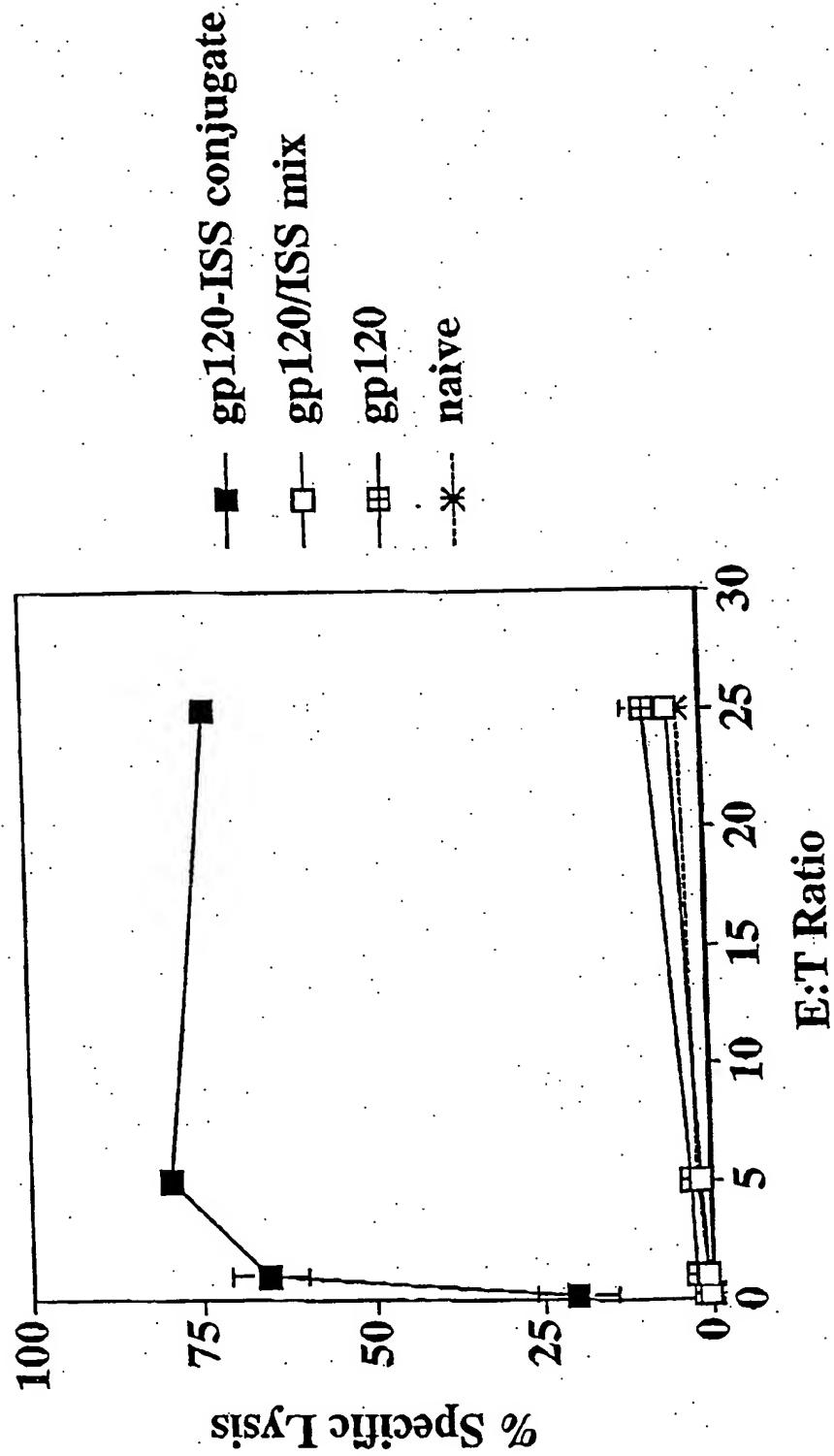


Figure 4

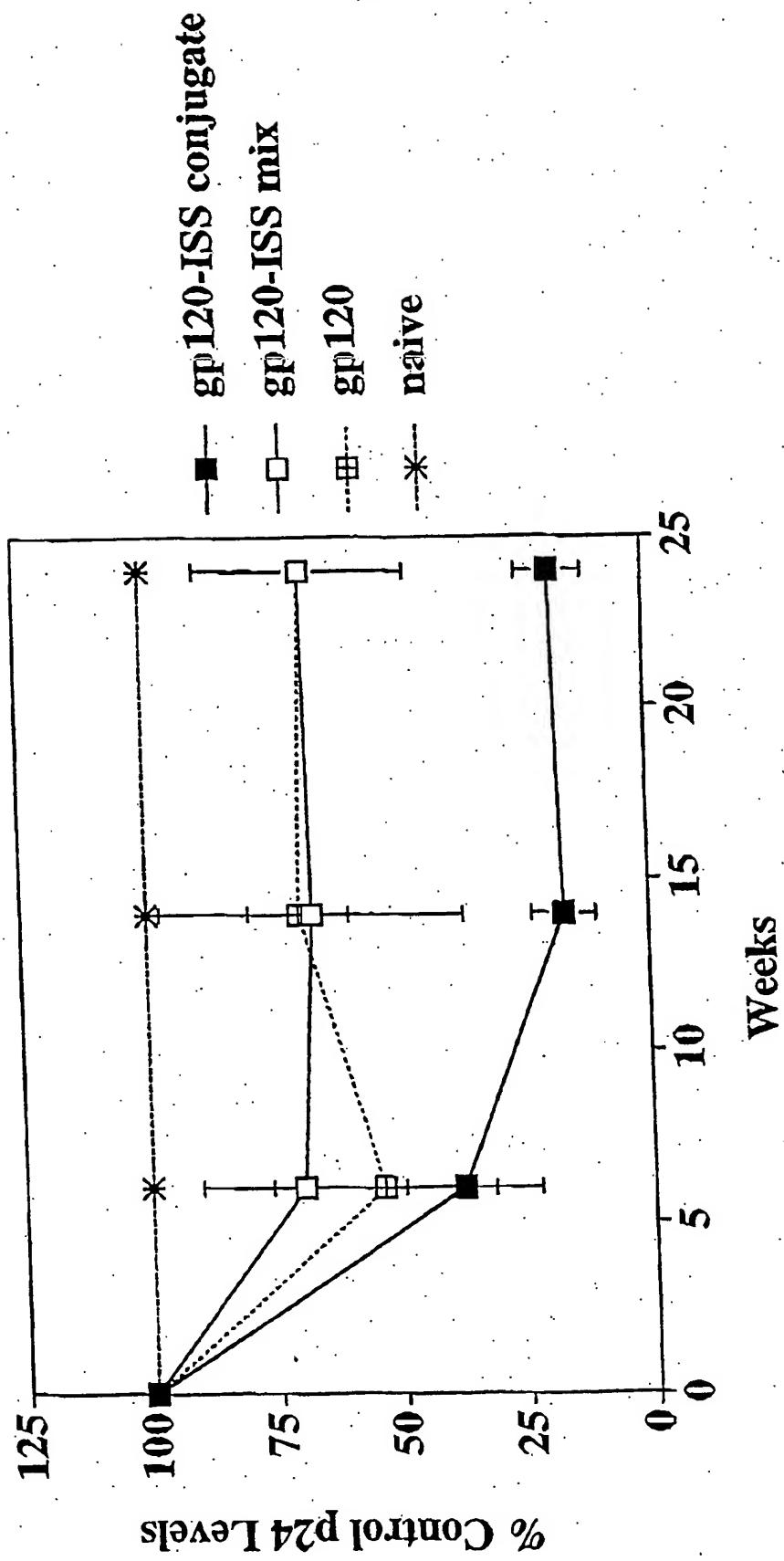


Figure 5

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/23677

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 16247 A (CARSON DENNIS A ; RAZ EYAL (US); ROMAN MARK (US); UNIV CALIFORNIA () 23 April 1998 (1998-04-23) the whole document, especially page 15 lines 1-3; page 18 lines 7-12; page 35-36 example 1; claims 1, 66 ---	1-43
X, P, L	WO 98 55495 A (DYNAVAX TECHNOLOGIES CORP ; DINA DINO (US); ROMAN MARK (US); SCHWAR) 10 December 1998 (1998-12-10) the whole document ---	1-43
E	WO 99 51259 A (UNIV IOWA RES FOUND) 14 October 1999 (1999-10-14) the whole document ---	1,12-14, 16,43 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

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10 March 2000

28/03/2000

Name and mailing address of the ISA  
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1

## INTERNATIONAL SEARCH REPORT

Int'l. Appl. No.

PCT/US 99/23677

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	DEMEL L ET AL: "IMMUNOSTIMULATORY CPG MOTIFS TRIGGER A T HELPER-1 IMMUNE RESPONSE TO HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) GP160 ENVELOPE PROTEINS" CLIN CHEM LAB MED, vol. 37, no. 3, March 1999 (1999-03), pages 199-204-204, XP000857051  the whole document ---	1,2,4,5, 7,9,10, 12-18, 20,21, 23-28, 30-34, 36, 38-40,43
A	WO 98 18810 A (UNIV IOWA RES FOUND ;KLINE JOEL N (US); KRIEG ARTHUR M (US)) 7 May 1998 (1998-05-07) the whole document ---	1-43
A	MICHAEL F. POWELL ET AL: "Vaccine Design- The subunit and adjuvant approach" 1996 , PLENUM PRESS , NEW YORK XP002132738 page 821 -page 845 -----	1-43

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 99/23677

**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark:** Although claims 12-43 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Intell. -nal Application No

PCT/US 99/23677

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9816247	A 23-04-1998	AU 4992197 A EP 0930893 A		11-05-1998 28-07-1999
WO 9855495	A 10-12-1998	AU 7811398 A AU 7817898 A WO 9855609 A		21-12-1998 21-12-1998 10-12-1998
WO 9951259	A 14-10-1999	AU 3467899 A		25-10-1999
WO 9818810	A 07-05-1998	AU 5242498 A CN 1235609 A EP 0948510 A		22-05-1998 17-11-1999 13-10-1999

**CORRECTED VERSION**

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
20 April 2000 (20.04.2000)

PCT

(10) International Publication Number  
**WO 00/21556 A1**

(51) International Patent Classification<sup>7</sup>: A61K 39/21 (81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(21) International Application Number: PCT/US99/23677 (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(22) International Filing Date: 8 October 1999 (08.10.1999)

(25) Filing Language: English

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(30) Priority Data:  
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09/415,186 7 October 1999 (07.10.1999) US

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4 October 2001

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see PCT Gazette No. 40/2001 of 4 October 2001, Section II

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 00/21556 A1

(54) Title: ANTI HIV COMPOSITIONS COMPRISING IMMUNOSTIMULATORY POLYNUCLEOTIDES AND HIV ANTI-GENS

(57) Abstract: The invention relates to anti-viral immunomodulatory compositions comprising immunostimulatory polynucleotides and HIV antigens, such as gp120. Methods for modulating an immune response upon administration of the oligonucleotide and antigen compositions are also disclosed.

## ANTI HIV COMPOSITIONS COMPRISING IMMUNOSTIMULATORY POLYNUCLEOTIDES AND HIV ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the priority benefit of U.S. Provisional application 60/103,733, filed October 9, 1998, which is hereby incorporated herein by reference in its entirety.

TECHNICAL FIELD

10 The present invention relates to immunomodulatory compositions comprising a viral antigen, particularly an HIV antigen, and further comprising an immunostimulatory polynucleotide sequence (ISS). The invention further relates to the administration of the compositions to modulate an immune response.

15

BACKGROUND ART

In spite of recent development of a number of treatments, such as protease cocktails, infection by human immunodeficiency virus (HIV) remains an extremely serious health threat worldwide. HIV infection causes depletion of the subset of T cells called T helper cells (or CD4<sup>+</sup> cells), which in turn severely compromises the immune system, 20 causing AIDS (acquired immune deficiency syndrome). Individuals suffering from AIDS are susceptible to a host of pathogenic infections and cancer.

The type of immune response generated to infection or other antigenic challenge can generally be distinguished by the subset of T helper (Th) cells involved in the response. The Th1 subset is responsible for classical cell-mediated functions such as delayed-type 25 hypersensitivity and activation of cytotoxic T lymphocytes (CTLs), whereas the Th2 subset functions more effectively as a helper for B-cell activation. The type of immune response to an antigen is generally determined by the cytokines produced by the cells responding to the antigen. Differences in the cytokines secreted by Th1 and Th2 cells are believed to reflect different biological functions of these two subsets.

30 The Th1 subset may be particularly suited to respond to viral infections and intracellular pathogens because it secretes IL-2 and IFN- $\gamma$ , which activate CTLs. The Th2

subset may be more suited to respond to free-living bacteria and helminthic parasites and may mediate allergic reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation, respectively. In general, Th1 and Th2 cells secrete distinct patterns of cytokines and so one type of response can moderate the activity of the other 5 type of response. A shift in the Th1/Th2 balance can result in an allergic response, for example, or, alternatively, in an increased CTL response.

Immunization of a host animal against a particular antigen has been accomplished traditionally by repeatedly vaccinating the host with an immunogenic form of the antigen. While most current vaccines elicit effective humoral (antibody, or "Th2-type") responses, 10 they fail to elicit cellular responses (in particular, major histocompatibility complex (MHC) class I-restricted CTL, or "Th1-type" responses) which are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, Th2-type responses are of little protective value against infection. Proposed vaccines using small peptides derived from the target antigen and other currently used antigenic agents that avoid use of potentially 15 infective intact viral particles, do not always elicit the immune response necessary to achieve a therapeutic effect. The lack of a therapeutically effective human immunodeficiency virus (HIV) vaccine is an unfortunate example of this failure. A recent review on the development of an HIV-1 vaccine suggested that for an HIV-1 vaccine to be effective, it must stimulate HIV-1-specific CTLs. Letvin (1998) *Science* 280:1875-1880.

20 Protein-based vaccines typically induce Th2-type immune responses, characterized by high titers of neutralizing antibodies but without significant cell-mediated immunity. In contrast, intradermal delivery of "naked", or uncomplexed, DNA encoding an antigen stimulates immune responses to the antigen with a Th1-type bias, characterized by the expansion of CD4<sup>+</sup> T cells producing IFN- $\gamma$  and cytotoxic CD8<sup>+</sup> T cells. Manickan et al. 25 (1995) *J. Immunol.* 155:250-265; Xiang et al. (1995) *Immunity* 2:129-135; Raz et al. (1995) *Proc. Natl. Acad. Sci. USA* 93:5141-5145; and Briode et al. (1997) *J. Allergy Clin. Immunol.* 99:s129. Injection of antigen-encoding naked DNA reproducibly induces both humoral and cellular immune responses against the encoded antigens. Pardoll and Beckerleg (1995) *Immunity* 3:165-169. DNA vaccines can provide a new approach to 30 infectious disease prophylaxis. See, for instance, Dixon (1995) *Bio/Technology* 13:420 and references cited therein.

Certain types of DNA, without being translated, have been shown to stimulate immune responses. Bacterial DNA induces anti-DNA antibodies in injected mice, as well as cytokine production by macrophage and natural killer (NK) cells. Pisetsky (1996a) *J. Immunol.* 156:421-423; Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Yamamoto et al. (1992a) *Microbiol. Immunol.* 36:983-897; and Cowdery et al. (1996) *J. Immunol.* 156:4570-4575.

B cell and NK cell activation properties of bacterial DNA have been associated with short (6 base pair hexamer) sequences that include a central unmethylated CpG dinucleotide. Yamamoto et al. (1992a); and Krieg et al. (1995) *Nature* 374:546-549. Oligonucleotides comprising a CpG sequence flanked by two 5' purines and two 3' pyrimidines have been shown to be most potent in B cell and NK cell stimulation. For example, when a variety of oligonucleotides comprising hexamers were tested for their ability to augment the NK cell activity of mouse spleen cells, the most immunogenic hexamers included AACGTT, AGCGCT, GACGTC. Yamamoto et al. (1992b) *J. Immunol.* 148:4072-4076. In a study in which B cell activation was measured in response to oligonucleotides, the most stimulatory hexamer sequences (e.g., AACGTC, AACGTT, GACGTC, GACGTT) also matched the sequence of 5'-purine, purine, CG, pyrimidine, pyrimidine-3'. Krieg et al. (1995). However, as shown herein, this prototypical hexamer sequence is found in many oligonucleotides that are not immunostimulatory. Thus, the prototypical hexamer sequence proposed by Krieg et al. (1995) is not predictive of immunostimulatory activity.

Bacterial DNA stimulated macrophages to produce IL-12 and TNF- $\alpha$ . These macrophage-produced cytokines were found to induce the production of IL-12 and IFN- $\gamma$  from splenocytes. Halpern et al. (1996) *Cell. Immunol.* 167:72-78. *In vitro* treatment of splenocytes with either bacterial DNA or CpG containing oligonucleotides induced the production of IL-6, IL-12 and IFN- $\gamma$ . Klinman et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:2879-2883. Production of all of these cytokines is indicative of induction of a Th1-type immune response rather than a Th2-type response.

To date, no clear consensus has been reached on the sequences both necessary and sufficient of immune stimulation. A recent study which examined induction of NK activity in response to CpG containing-oligonucleotides suggested that the unmethylated CpG motif was necessary but not sufficient for oligonucleotide induction of NK lytic activity. Ballas

et al. (1996) *J. Immunol.* 157:1840-1845. Sequences flanking the CpG appeared to influence the immunostimulatory activity of an oligonucleotide. Immunostimulatory activity of immunostimulatory sequences appears to be independent of adenosine-methylation, and whether the nucleotide is single or double-stranded. See, for example, 5 Tokunaga et al. (1989) *Microbiol. Immunol.* 33:929; Tokunaga et al. (1992) *Microbiol. Immunol.* 36:55-66; Yamamoto et al. (1992b); Messina et al. (1993) *Cell. Immunol.* 147:148-157; and Sato et al. (1996) *Science* 273:352-354. Oligonucleotide length also does not seem to be a factor, as double-stranded DNA 4 kb long (Sato et al. (1996)) or single-stranded DNA as short as 15 nucleotides in length (Ballas et al. (1996)) elicited immune 10 responses; though if oligonucleotide length was reduced below 8 bases or if the DNA was methylated with CpG methylase, immunostimulatory activity was abolished. Krieg et al. (1995).

Vaccination with certain DNA containing immunostimulatory motifs induces an 15 immune response with a Th1-type bias. For example, mice injected intradermally with *Escherichia coli* (*E. coli*)  $\beta$ -galactosidase ( $\beta$ -Gal) in saline or in the adjuvant alum responded by producing specific IgG1 and IgE antibodies, and CD4 $^{+}$  cells that secreted IL-4 and IL-5, but not IFN- $\gamma$ , demonstrating that the T cells were predominantly of the Th2 subset. However, mice injected intradermally (or with a tyne skin scratch applicator) with 20 plasmid DNA (in saline) encoding  $\beta$ -Gal and containing an ISS responded by producing IgG2a antibodies and CD4 $^{+}$  cells that secreted IFN- $\gamma$ , but not IL-4 and IL-5, demonstrating that the T cells were predominantly of the Th1 subset. Moreover, specific IgE production by the plasmid DNA-injected mice was reduced 66-75%. Raz et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:5141-5145. In general, the response to naked DNA immunization is characterized by production of IL-2, TNF $\alpha$  and IFN- $\gamma$  by antigen-stimulated CD4 $^{+}$  T cells, 25 which is indicative of a Th1-type response. This is particularly important in treatment of allergy and asthma as shown by the decreased IgE production.

In another example, the presence of an immunostimulatory sequence, such as the palindromic hexamer AACGTT, in an antigen-encoding plasmid vector injected 30 intradermally prompted the production of large amounts of IFN- $\alpha$ , IFN- $\beta$  and IL-12. Sato et al. (1996). IFN- $\alpha$  plays a role in the differentiation of naive T cells toward a Th1-type phenotype, antagonizes Th2 cells, inhibits IgE synthesis, promotes IgG2a production and

induces a Th1 phenotype of antigen-specific T cell clones. IL-12 promotes IFN- $\gamma$  production by T cells and favors maturation of Th1 cells.

It would be useful in treatment of a wide variety of indications to be able to specifically enhance the Th1-type response to a particular antigen while down-regulating the Th2-type response to the same antigen. Treatment or palliation of these indications includes, but is not limited to, induction of a vigorous cellular immune response and anti-viral therapy. The present invention provides compositions comprising oligonucleotide sequences that can be employed in these contexts.

Other references describing ISS include: Krieg et al. (1989) *J. Immunol.* 143:2448-2451; Tokunaga et al. (1992); Kataoka et al. (1992) *Jpn. J. Cancer Res.* 83:244-247; Yamamoto et al. (1992b); Mojcić et al. (1993) *Clin. Immuno. and Immunopathol.* 67:130-136; Branda et al. (1993) *Biochem. Pharmacol.* 45:2037-2043; Pisetsky et al. (1994) *Life Sci.* 54(2):101-107; Yamamoto et al. (1994a) *Antisense Research and Development.* 4:119-122; Yamamoto et al. (1994b) *Jpn. J. Cancer Res.* 85:775-779; Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523; Kimura et al. (1994) *J. Biochem. (Tokyo)* 116:991-994; Krieg et al. (1995); Pisetsky et al. (1995) *Ann. N.Y. Acad. Sci.* 772:152-163; Pisetsky (1996a); Pisetsky (1996b) *Immunity* 5:303-310; Zhao et al. (1996) *Biochem. Pharmacol.* 51:173-182; Yi et al. (1996) *J. Immunol.* 156:558-564; Krieg (1996) *Trends Microbiol.* 4(2):73-76; Krieg et al. (1996) *Antisense Nucleic Acid Drug Dev.* 6:133-139; Klinman et al. (1996); Raz et al. (1996); Sato et al. (1996); Stacey et al. (1996) *J. Immunol.* 157:2116-2122; Ballas et al. (1996); Branda et al. (1996) *J. Lab. Clin. Med.* 128:329-338; Sonehara et al. (1996) *J. Interferon and Cytokine Res.* 16:799-803; Klinman et al. (1997) *J. Immunol.* 158:3635-3639; Sparwasser et al. (1997) *Eur. J. Immunol.* 27:1671-1679; Roman et al. (1997); Carson et al. (1997) *J. Exp. Med.* 186:1621-1622; Chace et al. (1997) *Clin. Immunol. and Immunopathol.* 84:185-193; Chu et al. (1997) *J. Exp. Med.* 186:1623-1631; Lipford et al. (1997a) *Eur. J. Immunol.* 27:2340-2344; Lipford et al. (1997b) *Eur. J. Immunol.* 27:3420-3426; Weiner et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:10833-10837; Macfarlane et al. (1997) *Immunology* 91:586-593; Schwartz et al. (1997) *J. Clin. Invest.* 100:68-73; Stein et al. (1997) *Antisense Technology*, Ch. 11 pp. 241-264, C. Lichtenstein and W. Nellen, Eds., IRL Press; Wooldridge et al. (1997) *Blood* 89:2994-2998; Leclerc et al. (1997) *Cell. Immunol.* 179:97-106; Kline et al. (1997) *J. Invest. Med.* 45(3):282A; Yi et al. (1998a) *J. Immunol.* 160:1240-1245; Yi et al. (1998b) *J. Immunol.* 160:4755-4761; Yi et

al. (1998c) *J. Immunol.* 160:5898-5906; Yi et al. (1998d) *J. Immunol.* 161:4493-4497; Krieg (1998) *Applied Antisense Oligonucleotide Technology* Ch. 24, pp. 431-448, C.A. Stein and A.M. Krieg, Eds., Wiley-Liss, Inc.; Krieg et al. (1998a) *Trends Microbiol.* 6:23-27; Krieg et al. (1998b) *J. Immunol.* 161:2428-2434; Krieg et al. (1998c) *Proc. Natl. Acad. Sci. USA* 95:12631-12636; Spiegelberg et al. (1998) *Allergy* 53(45S):93-97; Horner et al. (1998) *Cell Immunol.* 190:77-82; Jakob et al. (1998) *J. Immunol.* 161:3042-3049; Redford et al. (1998) *J. Immunol.* 161:3930-3935; Weeratna et al. (1998) *Antisense & Nucleic Acid Drug Development* 8:351-356; McCluskie et al. (1998) *J. Immunol.* 161(9):4463-4466; Gramzinski et al. (1998) *Mol. Med.* 4:109-118; Liu et al. (1998) *Blood* 92:3730-3736; Moldoveanu et al. (1998) *Vaccine* 16: 1216-1224; Brazolot Milan et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:15553-15558; Briode et al. (1998) *J. Immunol.* 161:7054-7062; Briode et al. (1999) *Int. Arch. Allergy Immunol.* 118:453-456; Kovarik et al. (1999) *J. Immunol.* 162:1611-1617; Spiegelberg et al. (1999) *Pediatr. Pulmonol. Suppl.* 18:118-121; Martin-Orozco et al. (1999) *Int. Immunol.* 11:1111-1118; EP 468,520; WO 96/02555; WO 97/28259; WO 98/16247; WO 98/18810; WO 98/37919; WO 98/40100; WO 98/52581; WO 98/52962; WO 98/55495; WO 98/55609 and WO 99/11275. Elkins et al. (1999) *J. Immunol.* 162:2291-2298; WO 98/52962.

There remains a serious need to develop effective preventative and therapeutic strategies for HIV infection.

20

All of the cited literature included in the preceding section, as well as the cited literature included in the following disclosure, are hereby incorporated herein by reference.

#### DISCLOSURE OF THE INVENTION

25 The present invention provides immunomodulatory compositions comprising an antigen from human immunodeficiency virus (HIV) and further comprising an immunomodulatory polynucleotide, wherein the immunomodulatory polynucleotide comprises at least one immunostimulatory sequence (ISS). In some embodiments, the immunomodulatory polynucleotide is SEQ ID NO:1 (5'-  
30 TGACTGTGAAACGTTCGAGATGA-3'). In other embodiments, the immunomodulatory polynucleotide comprises SEQ ID NO:1.

In another embodiment, the immunomodulatory composition comprises an HIV polypeptide or glycoprotein. In some embodiments, the HIV antigen is a gp120 polypeptide.

5 In another embodiment, the immunomodulatory composition comprises the oligonucleotide SEQ ID NO:1 and the HIV gp120 antigen.

In some embodiments, the HIV antigen is conjugated to an immunomodulatory polynucleotide. In other embodiments, the HIV antigen is not conjugated to an immunomodulatory polynucleotide and is proximately associated with the immunomodulatory ISS-containing polynucleotide at a distance such that an immune 10 response is enhanced compared to co-administration of the immunomodulatory polynucleotide and HIV antigen in solution.

The invention also provides for methods of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory polynucleotide comprising an ISS and an HIV antigen. In some 15 embodiments, the immune response modulation comprises the induction of a Th1 response.

In some embodiments, the HIV antigen and the oligonucleotide are co-administered. In other embodiments, the HIV antigen is conjugated to an immunomodulatory polynucleotide comprising an ISS. In other embodiments, the HIV antigen is proximately associated with an immunomodulatory polynucleotide comprising an ISS at a distance such 20 that an immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and HIV antigen in solution.

Further embodiments include methods of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120 polypeptide and an immunomodulatory polynucleotide, wherein said polynucleotide comprises an immunostimulatory sequence (ISS), wherein a gp120 polypeptide is conjugated to the immunomodulatory polynucleotide, and wherein the immunomodulatory composition is administered to the individual in an amount sufficient to stimulate a specific immune response against gp120. The immune response may comprise production of an anti-gp120 antibody and/or production of T cells.

30 Also included are methods of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120 polypeptide and an immunomodulatory polynucleotide, wherein said

polynucleotide comprises an immunostimulatory sequence (ISS), wherein gp120 polypeptide is not conjugated and is proximately associated to the immunomodulatory polynucleotide at a distance such that an immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and gp120 polypeptide in solution, and wherein the immunomodulatory composition is administered to the individual in an amount sufficient to stimulate a specific immune response against gp120. The immune response may comprise production of an anti-gp120 antibody and/or production of T cells.

Further embodiments include methods of suppressing HIV infection in an individual infected with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection.

In some embodiments, the invention provides methods of delaying development of HIV infection in an individual at risk of infection with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection upon exposure to HIV.

The invention also provides for methods of treating an individual in need of immune modulation comprising administration of an immunomodulatory composition comprising an immunomodulatory oligonucleotide and an antigen, including, but not limited to, individuals suffering from an infectious disease. A further embodiment provides a method for treating individuals infected with human immunodeficiency virus.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the level of IFN- $\gamma$  found in the culture supernatant of splenocytes from mice injected with HIV gp120 compositions.

Figure 2 is a graph depicting serum levels of anti-gp120 IgG2a generated in treated animals.

Figure 3 is a graph depicting serum levels of anti-gp120 IgG1 generated in treated animals.

Figure 4 is a graph depicting anti-gp120 CTL responses from splenocytes of treated animals.

Figure 5 is a graph depicting levels of HIV neutralizing antibodies generated in treated animals.

#### MODES FOR CARRYING OUT THE INVENTION

5 The present invention provides compositions comprising a viral antigen, in particular an HIV antigen, and an immunomodulatory polynucleotide (used interchangeably herein with the term "oligonucleotide"), which, when administered to a host, stimulate an effective antibody and/or cellular response to said antigen. The invention also provides methods of modulating an immune response as well as methods that suppress or ameliorate HIV infection and/or stimulate an HIV-specific immune response which use 10 the compositions described herein.

#### General Techniques

15 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in 20 Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

25

#### Definitions

30 The term "ISS" as used herein refers to oligonucleotide sequences that effect a measurable immune response as measured *in vitro*, *in vivo* and/or *ex vivo*. Examples of measurable immune responses include, but are not limited to, antigen-specific antibody production, secretion of cytokines, activation or expansion of lymphocyte populations such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, B lymphocytes, and the like. Preferably, the ISS sequences preferentially activate a Th1-type response.

As used interchangeably herein, the terms "oligonucleotide" and "polynucleotide" include single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), modified oligonucleotides and oligonucleosides or combinations thereof. The oligonucleotide can be linearly or circularly configured, or the oligonucleotide can contain both linear and circular segments.

5 Oligonucleotides are polymers of nucleosides joined, generally, through phosphoester linkages. A nucleoside consists of a purine (adenine or guanine or derivative thereof) or pyrimidine (thymine, cytosine or uracil, or derivative thereof) base bonded to a sugar. The four nucleoside units (or bases) in DNA are called deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. A nucleotide is a phosphate ester of a nucleoside.

10 As used herein, the term "conjugate" refers to a complex in which an ISS and an immunomodulatory molecule are linked. Such conjugate linkages include covalent and/or non-covalent linkages.

15 As used herein, the term "antigen" means a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins, polysaccharides, gangliosides and lipids; portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic.

20 Haptens are included within the scope of "antigen." A hapten is a low molecular weight compound that is not immunogenic by itself but is rendered immunogenic when conjugated with an immunogenic molecule containing antigenic determinants. Antigens suitable for administration with ISS include any molecule capable of eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. A wide variety of molecules are antigens. These include, but are not limited to, sugars, lipids and polypeptides, as well as macromolecules such as complex carbohydrates, and 25 phospholipids. Small molecules may need to be haptenized in order to be rendered antigenic. Preferably, antigens of the present invention include peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *Hemophilus influenza* vaccines, gangliosides and glycoproteins.

30 As evident by the definition of "antigen", an "HIV antigen" is an antigen derived from a human immunodeficiency virus (HIV). These antigens may thus be, for example, polypeptides and glycoproteins (which may or may not consist of or comprise entire sequences or fragments). For simplicity, the term "HIV antigen" thus includes

polypeptides and glycoproteins, and a term such as "gp120 polypeptide" refers to glycoprotein embodiments, as one skilled in the art readily appreciates that gp120 is a glycoprotein.

5 As used herein, the term "adjuvant" refers to a substance which, when added to an immunogenic agent, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture.

10 As used herein, the term "peptide" includes peptides and proteins that are of sufficient length and composition to effect a biological response, e.g. antibody production or cytokine activity whether or not the peptide is a hapten. Typically, the peptides are of at least six amino acid residues in length. The term "peptide" further includes modified amino acids (whether or not naturally or non-naturally occurring) such modifications including, but not limited to, phosphorylation, glycosylation, pegylation, lipidization and methylation.

15 "Antigenic peptides" can include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-organisms, or fragments of such peptides. An "antigenic peptide" or "antigen polypeptide" accordingly means all or a portion of a polypeptide which exhibits one or more antigenic properties. Thus, a "gp120 antigenic polypeptide" or "gp120 polypeptide antigen" is an amino acid sequence from gp120, whether the entire sequence, a portion of the sequence, 20 and/or a modification of the sequence, which exhibits an antigenic property (i.e., binds specifically to an antibody or a T cell receptor).

25 The term "immunomodulatory" or "modulating an immune response" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, macrophages and the like; increased synthesis of immunostimulatory cytokines including, but not limited to, IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, IFN- $\gamma$ , TNF- $\alpha$  and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses. Examples of immunosuppressive effects include, 30 but are not limited to, a reduction in antigen-specific antibody production such as reduced

IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN- $\gamma$ , which appears to block IL-4 induced class switch to IgE and IgG1, thereby reducing the levels of these antibody subclasses.

5 As used herein, "in conjunction with" refers to administration of one treatment modality in addition to another treatment modality, such as administration of an ISS-containing polynucleotide to a subject in addition to the delivery of, for example, a protease inhibitor (or other treatment for HIV infection and/or AIDS) to the same subject, or 10 administration of two different ISS to the same subject. As such, "in conjunction with" refers to administration of one treatment modality before, during or after delivery of the other treatment modality to the subject.

15 As used herein, and as well-understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging 20 survival as compared to expected survival if not receiving treatment.

25 As used herein, "delaying" development of HIV infection means to defer, hinder, slow, retard, stabilize, and/or postpone development of the disease(s) associated with HIV infection when compared to not administering the ISS and HIV-antigen containing compositions described herein. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop disease(s) associated with HIV infection. A method that "delays" development of HIV infection is a method that reduces probability of disease development in a given time frame and/or reduces the extent of the disease in a given time frame, when compared to not using the method. Such comparisons are typically based on clinical studies, using a 30 statistically significant number of subjects.

An "individual" or "subject" is a vertebrate, preferably a mammal, and includes but is not limited to domestic animals, farm animals, sports animals, rodents, and primates, including humans.

5      *Compositions comprising ISS and antigen*

The invention provides compositions and methods useful for stimulating an anti-HIV immune response. A composition of the subject invention (any embodiment of which may be used in the methods described herein) comprises an HIV antigen and an ISS, and said composition is capable of eliciting a desired immune response. In some embodiments, 10 the invention provides compositions comprising ISS and one or more HIV antigenic peptides, such as gp120 polypeptide.

HIV antigens

15      Antigens used in the subject compositions and methods using the compositions are HIV antigens. Such antigens include, but are not limited to, those antigens derived from HIV envelope glycoproteins including, but not limited to, gp160, gp120 and gp41.

Generally, immunomodulatory peptides can be native or synthesized chemically or enzymatically. Any method of chemical synthesis known in the art is suitable. Solution phase peptide synthesis can be used to construct peptides of moderate size or, for the chemical construction of peptides, solid phase synthesis can be employed. Atherton et al. 20 (1981) *Hoppe Seylers Z. Physiol. Chem.* 362:833-839. Proteolytic enzymes can also be utilized to couple amino acids to produce peptides. Kullmann (1987) *Enzymatic Peptide Synthesis*, CRC Press, Inc. Alternatively, the peptide can be obtained by using the biochemical machinery of a cell, or by isolation from a biological source. Recombinant DNA techniques can be employed for the production of peptides. Hames et al. (1987) 25 *Transcription and Translation: A Practical Approach*, IRL Press. Peptides can also be isolated using standard techniques such as affinity chromatography. In certain cases, the peptides are commercially available.

30      Preferably the antigens are peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *H. influenza* vaccines, gangliosides and glycoproteins. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases,

such as for many sterols, fatty acids and phospholipids, the antigenic portions of the molecules are commercially available.

HIV antigens may be obtained using methods known in the art, for example, from native HIV virus extracts, from cells infected with HIV, from purified HIV polypeptides, 5 from recombinantly produced HIV polypeptides and/or as synthetic peptides. For example, purified HIV polypeptides from HIV infected cells are described in U.S. Patent No. 4,725,669. Polynucleotide sequences encoding HIV polypeptides may be cloned into expression vectors as described, for example in U.S. Patent No. 5,817,637, for isolation of polypeptides through the use of recombinant techniques. HIV polypeptides are also 10 commercially available, for example, from Intracel Corporation, Rockville, MD.

To generate HIV antigens, numerous sequences for HIV genes and antigens are known. For example, the Los Alamos National Laboratory HIV Sequence Database collects, curates and annotates HIV nucleotide and amino acid sequences. This database is accessible via the internet, at <http://hiv-web.lanl.gov/>, and in a yearly publication, see 15 *Human Retroviruses and AIDS Compendium* (for example, 1998 edition).

Examples of HIV nucleotide and amino acid sequences from a variety of HIV strains have been described. HIV polypeptides include: gag polypeptides, including membrane anchoring polypeptide p17, core capsid p24 and nucleocapsid proteins p7 and p6; polymerase polypeptides, including protease (p15), reverse transcriptase and RNase H 20 (p66/p51 heterodimer) and integrase (p31); envelope glycoproteins, including gp160, gp120 and gp41; vif polypeptide (p23); vpr polypeptide (p15); vpu polypeptide (p16); nef polypeptide (p27); rev polypeptide (p19) and tat polypeptide (p14). The HIV antigen of the present invention may comprise any HIV polypeptide, or derivative thereof, known in the art including, but not limited to, those described in Ratner et al. (1985) *Nature* 313:277-284; Muesing et al. (1985) *Nature* 313:450-458; Myers et al. (1992) *AIDS Res. Hum. Retroviruses* 8:373-386; McCutchan et al. (1992) *AIDS Res. Hum. Retroviruses* 8:1887-1895; Gурго et al. (1988) *Virol.* 164:531-536; Berman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5200-5204; Malim et al., 1989, *Cell* 58:205-214.

Amino acid sequences of many HIV peptides and polypeptides, including those of the polymerase polypeptides, are disclosed in U.S. Patent No. 5,843,638. Amino acid 30 sequences of HIV gag polypeptides and peptides, including those for antigens p24 and p17, are described, for example, in U.S. Patent Nos. 5,919,462; 5,859,193; 5,700,469 and

5,612,453. Amino acid sequences of HIV envelope polypeptides, including those for antigens gp41, gp160 and gp120, are described, for example, in U.S. Patent Nos. 5,859,193, 5,338,829 and 5,864,027. Amino acid sequences of HIV tat polypeptides are described, for example, in U.S. Patent No. 5,652,122.

5 In some embodiments, the HIV antigen is a surface glycoprotein, or functional fragment thereof (meaning that the fragment is able to specifically bind to an antibody or a T cell receptor). Accordingly, in some embodiments, the HIV antigen is a gp120 polypeptide (including gp120); a gp41 polypeptide (including gp41); a gp160 polypeptide (including gp160). As is evident to one skilled in the art, synthesis of a composition 10 comprising an ISS-containing polynucleotide and gp120 can lead to production of related compositions comprising other gp120 polypeptides (e.g., fragments and/or modified polypeptides) using well-developed methods known in the art. For example, a fragment (i.e., region or portion) of gp120, such as the CD4<sup>+</sup>-binding region, can be tested for its 15 ability to bind to an anti-gp120 antibody and/or a T cell receptor using standard binding assays. Alternatively, a fragment of gp120 can be tested for its ability to elicit a desired immune response when administered with an ISS-containing polynucleotide.

#### ISS

20 In accordance with the present invention, the immunomodulatory polynucleotide contains at least one ISS, and can contain multiple ISSs. The ISSs can be adjacent within the polynucleotide, or they can be separated by additional nucleotide bases within the polynucleotide.

25 ISS have been described in the art and may be readily identified using standard assays which indicate various aspects of the immune response, such as cytokine secretion, antibody production, NK cell activation and T cell proliferation. See, e.g., WO 97/28259; WO 98/16247; WO 99/11275; Krieg et al. (1995); Yamamoto et al. (1992); Ballas et al. (1996); Klinman et al. (1997); Sato et al. (1996); Pisetsky (1996a); Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Cowdery et al. (1996) *J. Immunol.* 156:4570-4575; Roman et al. (1997); and Lipford et al. (1997a).

30 The ISS can be of any length greater than 6 bases or base pairs and generally comprises the sequence 5'-cytosine, guanine-3', more particularly comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3' (such as 5'-AACGTT-3'), preferably greater than 15 bases or base pairs, more preferably greater than 20 bases or base pairs in

length. In some embodiments, the ISS comprises the sequence 5'-purine, T, C, G, pyrimidine, pyrimidine-3'. In some embodiments, the oligonucleotide of the composition contains at least one octameric ISS. It has been found that a particular set of octanucleotide sequences within oligonucleotide sequences renders the oligonucleotide capable of 5 modulating an immune response. Such oligonucleotide sequences comprise an immunostimulatory octanucleotide sequence (ISS). The ISS of the present invention comprise an octanucleotide sequence which comprises the previously described hexamer and two additional nucleotides 3' of the hexamer. The octameric ISS preferably comprises a CG containing sequence of the general octameric sequence 5'-Purine, Purine, Cytosine, 10 Guanine, Pyrimidine, Pyrimidine, Cytosine, (Cytosine or Guanine)-3'. Most preferably, the ISS comprises an octamer selected from the group consisting of: AACGTTCC, AACGTTCG, GACGTTCC, and GACGTTCG. Where the immunostimulatory oligonucleotide comprises an RNA sequence, the ISS preferably comprises a single-stranded or double-stranded sequence selected from the group consisting of AACGUUCC, 15 AACGUUCG, GACGUUCC, and GACGUUCG.

Accordingly, an ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. An ISS may also comprise the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'.

In some embodiments, the ISS comprises any of the following sequences: 20 GACGCTCC; GACGTCCC; GACGTTCC; GACGCCCC; AGCGTTCC; AGCGCTCC; AGCGTCCC; AGCGCCCC; AACGTCCC; AACGCCCC; AACGTTCC; AACGCTCC; GGCGTTCC; GGCGCTCC; GGCGTCCC; GGCGCCCC; GACGCTCG; GACGTCCG; GACGCCCG; GACGCTCG; AGCGCTCG; AGCGTTCG; AGCGTCCG; AGCGCCCCG; AACGTCCG; AACGCCCG; AACGTTCG; AACGCTCG; GGCGTTCG; GGCGCTCG; 25 GGCGTCCG; GGCGCCCCG. In some embodiments, the immunomodulatory polynucleotide comprises the sequence 5'-TGACTGTGAACGTTCGAGATGA-3' (SEQ ID NO:1). In other embodiments, the ISS comprises any of the sequences: 5'-TGACCGTGAAACGTTCGAGATGA-3'; 5'-TCATCTCGAACGTTCCACAGTCA-3'; 30 5'-TGACTGTGAACGTTCCAGATGA-3'; 5'-TCCATAACGTTCGCCTAACGTTCGTC-3'; 5'-TGACTGTGAABGTTCCAGATGA-3', where B is 5-bromocytosine;

5'-TGACTGTGAABGTCGAGATGA-3', where B is 5-bromocytosine and  
5'-TGACTGTGAABGTTBGAGATGA-3', where B is 5-bromocytosine.

An ISS and/or ISS-containing polynucleotide may contain modifications.

Modifications of ISS include any known in the art, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. Various such modifications are described below. Multiple bases, sugars, or phosphates in any combination can be substituted in the ISS.

An ISS may be single stranded or double stranded DNA, as well as single or double-stranded RNA or other modified polynucleotides. In general, dsRNA exerts an immunostimulatory effect and is encompassed by the invention.

An ISS may or may not include one or more palindromic regions, which may be present in the hexameric motif described above or may extend beyond the motif. An ISS may comprise additional flanking sequences, some of which are described herein. An ISS may contain naturally-occurring or modified, non-naturally occurring bases, and may contain modified sugar, phosphate, and/or termini. For example, phosphate modifications include, but are not limited to, methyl phosphonate, phosphorothioate, phosphoramidate (bridging or non-bridging), phosphotriester and phosphorodithioate and may be used in any combination. Other non-phosphate linkages may also be used. Preferably, oligonucleotides of the present invention comprise phosphorothioate backbones. Sugar modifications known in the field, such as 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras and others described herein, may also be made and combined with any phosphate modification. Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS (e.g., 5-bromocytosine, 5-chlorocytosine, 5-fluorocytosine, 5-iodocytosine). When the same cytosine is methylated, all immunostimulatory activity of the oligonucleotide is lost. Preferably, in such compositions, the cytosine in the third position from the 5' end can be substituted with a cytosine analog, preferably 5-bromocytidine, fluorinated cytosine, or chlorinated cytosine. Some of the modified ISS have approximately the same, if not greater, immunostimulatory activity relative to the same sequence without a modified base.

The ISS-containing oligonucleotide can comprise any other physiologically acceptable modified nucleotide base.

The ISS can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, Ausubel et al. (1987); and Sambrook et al. (1989). When assembled enzymatically, the individual units can be ligated, for example, with a ligase such as T4 DNA or RNA ligase. U.S. Patent No. 5,124,246. Oligonucleotide degradation can be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675.

The ISS can also be isolated using conventional polynucleotide isolation procedures. Such procedures include, but are not limited to, hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences, antibody screening of expression libraries to detect shared structural features and synthesis of particular native sequences by the polymerase chain reaction.

Circular ISS can be isolated, synthesized through recombinant methods, or chemically synthesized. Where the circular ISS is obtained through isolation or through recombinant methods, the ISS will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using any method described in the literature. See, for instance, Gao et al. (1995) *Nucleic Acids Res.* 23:2025-2029; and Wang et al. (1994) *Nucleic Acids Res.* 22:2326-2333.

The techniques for making oligonucleotides and modified oligonucleotides are known in the art. Naturally occurring DNA or RNA, containing phosphodiester linkages, is generally synthesized by sequentially coupling the appropriate nucleoside phosphoramidite to the 5'-hydroxy group of the growing oligonucleotide attached to a solid support at the 3'-end, followed by oxidation of the intermediate phosphite triester to a phosphate triester. Once the desired oligonucleotide sequence has been synthesized, the oligonucleotide is removed from the support, the phosphate triester groups are deprotected to phosphate diesters and the nucleoside bases are deprotected using aqueous ammonia or other bases. See, for example, Beaucage (1993) "Oligodeoxyribonucleotide Synthesis" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, Totowa, NJ; Warner et al. (1984) *DNA* 3:401 and U.S. Patent No. 4,458,066.

The ISS can also contain phosphate-modified oligonucleotides. Synthesis of polynucleotides containing modified phosphate linkages or non-phosphate linkages is also known in the art. For a review, see Matteucci (1997) "Oligonucleotide Analogs: an Overview" in *Oligonucleotides as Therapeutic Agents*, (D.J. Chadwick and G. Cardew, ed.) 5 John Wiley and Sons, New York, NY. The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the oligonucleotides of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphonate, phosphorothioate, phosphorodithioate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified 10 nucleotides and oligonucleotides, *per se*, is also known and need not be described here in detail. Peyrottes et al. (1996) *Nucleic Acids Res.* 24:1841-1848; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz et al. (1996) *Nucleic Acids Res.* 24:2966-2973. For example, synthesis of phosphorothioate oligonucleotides is similar to that 15 described above for naturally occurring oligonucleotides except that the oxidation step is replaced by a sulfurization step (Zon (1993) "Oligonucleoside Phosphorothioates" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, pp. 165-190). Similarly the synthesis of other phosphate analogs, such as phosphotriester (Miller et al. (1971) *JACS* 93:6657-6665), non-bridging phosphoramidates (Jager et al. (1988) *Biochem.* 27:7247-7246), N3' to P5' phosphoramidates (Nelson et al. 20 (1997) *JOC* 62:7278-7287) and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used (Stirchak et al. (1989) *Nucleic Acids Res.* 17:6129-6141). Oligonucleotides with phosphorothioate backbones can be more immunogenic than those with phosphodiester backbones and appear to be more resistant to degradation after injection into the host. 25 Braun et al. (1988) *J. Immunol.* 141:2084-2089; and Latimer et al. (1995) *Mol. Immunol.* 32:1057-1064.

The oligonucleotide of the invention can comprise ribonucleotides (containing 30 ribose as the only or principal sugar component), deoxyribonucleotides (containing deoxyribose as the principal sugar component), or, as is known in the art, modified sugars or sugar analogs can be incorporated in the ISS. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar can

be in pyranosyl or in a furanosyl form. In the ISS, the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-0-alkylribose, and the sugar can be attached to the respective heterocyclic bases either in  $\alpha$  or  $\beta$  anomeric configuration. Sugar modifications include, but are not limited to, 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) *per se* is known, and need not be described here, except to the extent such preparation can pertain to any specific example. Sugar modifications may also be made and combined with any phosphate modification in the preparation of an ISS.

The heterocyclic bases, or nucleic acid bases, which are incorporated in the ISS can be the naturally-occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally-occurring and synthetic modifications of said principal bases.

Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the present invention are satisfied, the ISS can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, the heterocyclic base in the ISS includes, but is not limited to, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the ISS via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

The ISS may comprise at least one modified base. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the ISS. Preferably, the electron-withdrawing moiety is a halogen. Such modified cytosines can include, but are not limited to,

azacytosine, 5-bromocytosine, bromouracil, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, 5-fluorocytosine, fluoropyrimidine, fluorouracil, 5,6-dihydrocytosine, 5-iodocytosine, hydroxyurea, iodouracil, 5-nitrocytosine, uracil, and any other pyrimidine analog or modified pyrimidine.

5 The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, 10 present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also been described (including, but not limited to, e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

15 In some embodiments, an ISS-containing polynucleotide is less than about any of the following lengths (in bases or base pairs): 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; 10. In some embodiments, an ISS-containing polynucleotide is greater than about any of the following lengths (in bases or base pairs): 8; 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500; 10000; 20000; 50000.

20 **ISS-HIV antigen**

The ISS-containing polynucleotide-HIV antigen compositions described herein may be in a number of configurations. Generally, the ISS-containing polynucleotide may be in an admixture (i.e., in solution) or proximately associated to each other by any of a number of means as described below. In some embodiments, the HIV antigen, such as gp120 polypeptide, is conjugated to the ISS-containing polynucleotide. In some embodiments, the HIV antigen is proximately associated with the ISS-containing polynucleotide (and is not conjugated). As described below, spatial proximation (proximate association) can be accomplished in a number of ways, including encapsidation, via affixation to a platform or adsorption onto a surface. Generally, and most preferably, an ISS-containing polynucleotide and HIV antigen are proximately associated at a distance effective to enhance the immune response generated compared to the administration of the ISS and HIV antigen as an admixture. In some embodiments, HIV antigen and ISS-containing

polynucleotide are in an admixture (i.e., in solution). If in an admixture (i.e., solution) the ISS and antigen are maintained at concentrations effective to modulate an immune response.

Accordingly, the invention provides compositions (and methods using these compositions) in which the HIV antigen(s) is conjugated to an ISS-containing polynucleotide. Preferably, the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'. Preferably, the ISS comprises or consists of SEQ ID NO:1. The HIV antigen may be any of the antigens described above, and preferably is a gp120 polypeptide (which includes gp120).

The ISS portion can be coupled with the antigen portion of a conjugate in a variety of ways, including covalent and/or non-covalent interactions.

The link between the portions can be made at the 3' or 5' end of the ISS, or at a suitably modified base at an internal position in the ISS. If the antigen is a peptide and contains a suitable reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N<sup>4</sup> amino group of cytosine residues. Depending on the number and location of cytosine residues in the ISS, specific coupling at one or more residues can be achieved.

Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the antigen of interest.

Where the antigen is a peptide, this portion of the conjugate can be attached to the 3'-end of the ISS through solid support chemistry. For example, the ISS portion can be added to a polypeptide portion that has been pre-synthesized on a support. Haralambidis et al. (1990a) *Nucleic Acids Res.* 18:493-499; and Haralambidis et al. (1990b) *Nucleic Acids Res.* 18:501-505. Alternatively, the ISS can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the ISS from the support, a terminal thiol group is left at the 3'-end of the oligonucleotide (Zuckermann et al. (1987) *Nucleic Acids Res.* 15:5305-5321; and Corey et al. (1987) *Science* 238:1401-1403) or a terminal amino group is left at the 3'-end of the oligonucleotide (Nelson et al. (1989) *Nucleic Acids Res.* 17:1781-1794). Conjugation of the amino-modified ISS to amino groups of the peptide can be performed as described in

Benoit et al. (1987) *Neuromethods* 6:43-72. Conjugation of the thiol-modified ISS to carboxyl groups of the peptide can be performed as described in Sinah et al. (1991) *Oligonucleotide Analogues: A Practical Approach*, IRL Press. Coupling of an oligonucleotide carrying an appended maleimide to the thiol side chain of a cysteine residue of a peptide has also been described. Tung et al. (1991) *Bioconjug. Chem.* 2:464-465.

The peptide portion of the conjugate can be attached to the 5'-end of the ISS through an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis. Preferably, while the oligonucleotide is fixed to the solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl. Agrawal et al. (1986) *Nucleic Acids Res.* 14:6227-6245; Connolly (1985) *Nucleic Acids Res.* 13:4485-4502; Kremsky et al. (1987) *Nucleic Acids Res.* 15:2891-2909; Connolly (1987) *Nucleic Acids Res.* 15:3131-3139; Bischoff et al. (1987) *Anal. Biochem.* 164:336-344; Blanks et al. (1988) *Nucleic Acids Res.* 16:10283-10299; and U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802. Subsequent to deprotection, the amine, thiol, and carboxyl functionalities can be used to covalently attach the oligonucleotide to a peptide. Benoit et al. (1987); and Sinah et al. (1991).

An ISS-antigen conjugate can also be formed through non-covalent interactions, such as ionic bonds, hydrophobic interactions, hydrogen bonds and/or van der Waals attractions.

Non-covalently linked conjugates can include a non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an ISS. Roget et al. (1989) *Nucleic Acids Res.* 17:7643-7651. Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated oligonucleotide.

Non-covalent associations can also occur through ionic interactions involving an ISS and residues within the antigen, such as charged amino acids, or through the use of a linker portion comprising charged residues that can interact with both the oligonucleotide and the antigen. For example, non-covalent conjugation can occur between a generally negatively-charged ISS and positively-charged amino acid residues of a peptide, e.g., polylysine, polyarginine and polyhistidine residues.

Non-covalent conjugation between ISS and antigens can occur through DNA binding motifs of molecules that interact with DNA as their natural ligands. For example, such DNA binding motifs can be found in transcription factors and anti-DNA antibodies.

5 The linkage of the ISS to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al. (1988) *Nucleic Acids Symp. Ser.* 19:189-192), oligonucleotide-fatty acid conjugates (Grabarek et al. (1990) *Anal. Biochem.* 185:131-135; and Staros et al. (1986) *Anal. Biochem.* 156:220-222), and oligonucleotide-sterol conjugates. Boujrad et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5728-5731.

10 The linkage of the oligonucleotide to an oligosaccharide can be formed using standard known methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin. O'Shannessy et al. (1985) *J. Applied Biochem.* 7:347-355.

15 The linkage of a circular ISS to a peptide or antigen can be formed in several ways. Where the circular ISS is synthesized using recombinant or chemical methods, a modified nucleoside is suitable. Ruth (1991) in *Oligonucleotides and Analogues: A Practical Approach*, IRL Press. Standard linking technology can then be used to connect the circular ISS to the antigen or other peptide. Goodchild (1990) *Bioconjug. Chem.* 1:165. Where the circular ISS is isolated, or synthesized using recombinant or chemical methods, the linkage 20 can be formed by chemically activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or other peptide.

25 Additional methods for the attachment of peptides and other molecules to oligonucleotides can be found in U.S. Patent No. 5,391,723; Kessler (1992) "Nonradioactive labeling methods for nucleic acids" in Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press; and Geoghegan et al. (1992) *Bioconjug. Chem.* 3:138-146.

30 The peptide portion can be attached to a modified cytosine or uracil at any position in the ISS. The incorporation of a "linker arm" possessing a latent reactive functionality, such as an amine or carboxyl group, at C-5 of the modified base provides a handle for the peptide linkage. Ruth, *4th Annual Congress for Recombinant DNA Research*, p. 123.

The invention also provides for compositions which comprise an ISS-antigen conjugate or an ISS-antigen admixture and an adjuvant where, upon co-administration, the

association of ISS-antigen and adjuvant is effective to enhance an immune response compared to the co-administration of the ISS-antigen without adjuvant. In such compositions, the adjuvant is maintained in association with ISS-antigen so as to recruit and activate target cells to the ISS-antigen.

5 The invention further provides for compositions in which ISS and an antigen are in proximate association at a distance effective to enhance the immune response generated compared to the administration of the ISS and the antigen as an admixture (i.e., compared to the co-administration of the ISS and antigen in solution). In some embodiments, the proximate association is by means other than conjugation. Preferably, the ISS and antigen are proximately associated at a distance of about 0.04  $\mu$ m to about 100  $\mu$ m, more preferably, at a distance of about 0.1  $\mu$ m to about 20  $\mu$ m, even more preferably, at a distance of about 0.15  $\mu$ m to about 10  $\mu$ m. Targets of the ISS-antigen conjugate or the ISS-antigen admixture include, but are not limited to, antigen presenting cells (APCs), such as macrophages, dendritic cells, and/or lymphocytes, lymphatic structures, such as lymph nodes and/or the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found, such as skin, lungs, and/or gastrointestinal tract.

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Enhancement of an immune response by a composition in which an ISS and an immunomodulatory agent are proximately associated refers to a modulation of an immune response following administration of said composition as compared to the immune response following administration of the ISS and immunomodulatory agent freely soluble with respect to each other. Enhancement of an immune response includes modulation of an immune response including, but not limited to, stimulation, suppression and a shift in the type of immune response, for instance, between a Th1-type response and a Th2-type response.

20

25 An ISS may be proximately associated with an antigen(s) in a number ways. In some embodiments, an ISS and antigen are proximately associated by encapsulation. In other embodiments, an ISS and antigen are proximately associated by linkage to a platform molecule. A "platform molecule" (also termed "platform") is a molecule containing sites which allow for attachment of the ISS and antigen(s). In other embodiments, an ISS and antigen are proximately associated by adsorption onto a surface, preferably a carrier 30 particle.

In some embodiments, the methods of the invention employ an encapsulating agent that can maintain the proximate association of the ISS and HIV antigen until the complex is available to the target (or compositions comprising such encapsulating agents). Preferably, the composition comprising ISS, antigen and encapsulating agent is in the form of adjuvant oil-in-water emulsions, microparticles and/or liposomes. More preferably, adjuvant oil-in-water emulsions, microparticles and/or liposomes encapsulating an ISS-immunomodulatory molecule are in the form of particles from about 0.04  $\mu$ m to about 100  $\mu$ m in size, preferably any of the following ranges: from about 0.1  $\mu$ m to about 20  $\mu$ m; from about 0.15  $\mu$ m to about 10  $\mu$ m; from about 0.05  $\mu$ m to about 1.00  $\mu$ m; from about 0.05  $\mu$ m to 10

5 about 0.5  $\mu$ m.

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Colloidal dispersion systems, such as microspheres, beads, macromolecular complexes, nanocapsules and lipid-based system, such as oil-in-water emulsions, micelles, mixed micelles and liposomes can provide effective encapsulation of ISS-containing compositions.

15 The encapsulation composition further comprises any of a wide variety of components. These include, but are not limited to, alum, lipids, phospholipids, lipid membrane structures (LMS), polyethylene glycol (PEG) and other polymers, such as polypeptides, glycopeptides, and polysaccharides.

20 Polypeptides suitable for encapsulation components include any known in the art and include, but are not limited to, fatty acid binding proteins. Modified polypeptides contain any of a variety of modifications, including, but not limited to glycosylation, phosphorylation, myristylation, sulfation and hydroxylation. As used herein, a suitable polypeptide is one that will protect an ISS-containing composition to preserve the immunomodulatory activity thereof. Examples of binding proteins include, but are not limited to, albumins such as bovine serum albumin (BSA) and pea albumin.

25

Other suitable polymers can be any known in the art of pharmaceuticals and include, but are not limited to, naturally-occurring polymers such as dextrans, hydroxyethyl starch, and polysaccharides, and synthetic polymers. Examples of naturally occurring polymers include proteins, glycopeptides, polysaccharides, dextran and lipids. The 30 additional polymer can be a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as PEG, polyoxyethylated polyols (POP), such as polyoxyethylated glycerol

(POG), polytrimethylene glycol (PTG) polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone (PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, co-polymers, or block co-polymers of two or more different synthetic monomers.

5 PEGs constitute a diverse group of molecules. A general formula for PEGs is as follows:



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where  $R_1$  and  $R_3$  are independently H,  $H_3C$ , OH, or a linear or branched, substituted or unsubstituted alkyl group and  $n$  is an integer between 1 and about 1,000. The term "PEG" includes both unsubstituted ( $R_1$  and  $R_3$  = H) as well as substituted PEG. The PEGs for use in encapsulation compositions of the present invention are either purchased from 15 chemical suppliers or synthesized using techniques known to those of skill in the art.

15 The term "LMS", as used herein, means lamellar lipid particles wherein polar head groups of a polar lipid are arranged to face an aqueous phase of an interface to form membrane structures. Examples of the LMSs include liposomes, micelles, cochleates (i.e., generally cylindrical liposomes), microemulsions, unilamellar vesicles, multilamellar 20 vesicles, and the like.

25 A preferred colloidal dispersion system of this invention is a liposome. In mice immunized with a liposome-encapsulated antigen, liposomes appeared to enhance a Th1-type immune response to the antigen. Aramaki et al. (1995) *Vaccine* 13:1809-1814. As used herein, a "liposome" or "lipid vesicle" is a small vesicle bounded by at least one and possibly more than one bilayer lipid membrane. Liposomes are made artificially from phospholipids, glycolipids, lipids, steroids such as cholesterol, related molecules, or a combination thereof by any technique known in the art, including but not limited to sonication, extrusion, or removal of detergent from lipid-detergent complexes. A liposome can also optionally comprise additional components, such as a tissue targeting component. 30 It is understood that a "lipid membrane" or "lipid bilayer" need not consist exclusively of lipids, but can additionally contain any suitable other components, including, but not limited to, cholesterol and other steroids, lipid-soluble chemicals, proteins of any length,

and other amphipathic molecules, providing the general structure of the membrane is a sheet of two hydrophilic surfaces sandwiching a hydrophobic core. For a general discussion of membrane structure, see *The Encyclopedia of Molecular Biology* by J. Kendrew (1994). For suitable lipids see e.g., Lasic (1993) "Liposomes: from Physics to Applications" Elsevier, Amsterdam.

5 Preferably, a liposomal composition is chosen that allows the membrane to be formed with reproducible qualities, such as diameter, and is stable in the presence of elements expected to occur where the liposome is to be used, such as physiological buffers and circulating molecules. Preferably, the liposome is resilient to the effects of manipulation by storage, freezing, and mixing with pharmaceutical excipients.

10 Lipids suitable for incorporation into lipid membrane structures include, but are not limited to, natural, semi-synthetic or synthetic mono- or di-glycerophospholipids including, but not limited to, phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylglycerols (PGs), phosphatidylinositols (PIs), phosphatidic acids (PAs), phosphatidylserines (PSs), glycero- and cardiolipins. Sphingolipids such as sphingomyelin (SM) and cerebrosides can also be incorporated. While natural phospholipids occur with the phospho moiety at the *sn*-3 position and hydrophobic chains at the *sn*-1 and *sn*-2 positions, synthetic lipids can have alternative stereochemistry with, e.g., the phospho group at the *sn*-1 or *sn*-2 positions. Furthermore, the hydrophobic chains can be attached to the glycerol backbone by acyl, ether, alkyl or other linkages. Derivatives of these lipids are 15 also suitable for incorporation into liposomes. Derivatives suitable for use include, but are not limited to, haloalkyl derivatives, including those in which all or some of the hydrogen atoms of the alkyl chains are substituted with, e.g., fluorine. In addition, cholesterol and other amphipathic steroids, bolaamphiphiles (lipids with polar moieties at either end of the molecule which form monolayer membranes) and polyglycerolmonoalkylthers can also be 20 incorporated. Liposomes can be composed of a single lipid or mixtures of two or more different lipids.

25 In one embodiment, the lipid bilayer of the liposome is formed primarily from phospholipids. Preferably, the phospholipid composition is a complex mixture, comprising a combination of PS and additional lipids such as PC, PA, PE, PG and SM, PI, and/or cardiolipin (diphosphatidylglycerol). If desired, SM can be replaced with a greater proportion of PC, PE, or a combination thereof. PS can be optionally replaced with PG.

The composition is chosen so as to confer upon the LMS both stability during storage and administration.

Practitioners of ordinary skill will readily appreciate that each phospholipid in the foregoing list can vary in its structure depending on the fatty acid moieties that are esterified to the glycerol moiety of the phospholipid. Generally, most commercially available forms of a particular phospholipid can be used. However, phospholipids containing particular fatty acid moieties may be preferred for certain applications.

Processes for preparing liposomes containing ISS-containing compositions are known in the art. The lipid vesicles can be prepared by any suitable technique known in the art. Methods include, but are not limited to, microencapsulation, microfluidization, LLC method, ethanol injection, freon injection, the "bubble" method, detergent dialysis, hydration, sonication, and reverse-phase evaporation. Reviewed in Watwe et al. (1995) *Curr. Sci.* 68:715-724. Techniques may be combined in order to provide vesicles with the most desirable attributes.

A general process for preparing liposomes containing ISS and antigen-containing compositions is as follows. An aqueous dispersion of liposomes is prepared from membrane components, such as phospholipids (e.g. PS, PC, PG, SM and PE) and glycolipids according to any known methods. See, e.g., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980). The liposomes can further contain sterols, dialkylphosphates, diacylphosphatidic acids, stearylamine,  $\alpha$ -tocopherol, etc., in the liposomal membrane.

To the liposomal dispersion thus prepared is added an aqueous solution of the ISS and antigen-containing composition and the mixture is allowed to stand for a given period of time, preferably under warming at a temperature above the phase transition temperature of the membrane or above 40°C, followed by cooling to thereby prepare liposomes containing the ISS and antigen-containing composition in the liposomal membrane.

Alternatively, the desired liposomes can also be prepared by previously mixing the above-described membrane components and ISS and antigen-containing composition and treating the mixture in accordance with known methods for preparing liposomes.

Optionally, the LMS also includes steroids to improve the rigidity of the membrane. Any amount of a steroid can be used. Suitable steroids include, but are not limited to, cholesterol and cholestanol. Other molecules that can be used to increase the rigidity of the membrane include, but are not limited to, cross-linked phospholipids.

Other preferred LMSs for use *in vivo* are those with an enhanced ability to evade the reticuloendothelial system, which normally phagocytoses and destroys non-native materials, thereby giving the liposomes a longer period in which to reach the target cell. Effective lipid compositions in this regard are those with a large proportion of SM and cholesterol, or SM and PI. LMSs with prolonged circulation time also include those that comprise the monosialoganglioside GM1, glucuronide, or PEG.

The invention encompasses use of LMSs containing tissue or cellular targeting components. Such targeting components are components of a LMS that enhance its accumulation at certain tissue or cellular sites in preference to other tissue or cellular sites when administered to an intact animal, organ, or cell culture. A targeting component is generally accessible from outside the liposome, and is therefore preferably either bound to the outer surface or inserted into the outer lipid bilayer. A targeting component can be *inter alia* a peptide, a region of a larger peptide, an antibody specific for a cell surface molecule or marker, or antigen binding fragment thereof, a nucleic acid, a carbohydrate, a region of a complex carbohydrate, a special lipid, or a small molecule such as a drug, hormone, or hapten, attached to any of the aforementioned molecules. Antibodies with specificity toward cell type-specific cell surface markers are known in the art and are readily prepared by methods known in the art.

The LMSs can be targeted to any cell type toward which a therapeutic treatment is to be directed, e.g., a cell type which can modulate and/or participate in an immune response. Such target cells and organs include, but are not limited to, APCs, such as macrophages, dendritic cells and lymphocytes, lymphatic structures, such as lymph nodes and the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found.

The LMS compositions of the present invention can additionally comprise surfactants. Surfactants can be cationic, anionic, amphiphilic, or nonionic. A preferred class of surfactants are nonionic surfactants; particularly preferred are those that are water soluble. Nonionic, water soluble surfactants include polyoxyethylene derivatives of fatty alcohols, fatty acid ester of fatty alcohols and glyceryl esters, wherein the polyoxyethylene group is coupled via an ether linkage to an alcohol group. Examples include, but are not limited to, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil

derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyoxyethylene fatty acid ester and polyoxyethylene alkyl ethers.

The LMS compositions encompassed herein include micelles. The term "micelles" as used herein means aggregates which form from tenside molecules in aqueous solutions above a specific temperature (Krafft point) or a characteristic concentration, the critical micellization concentration (cmc). When the cmc is exceeded, the monomer concentration remains practically constant and the excess tenside molecules form micelles. Micelles are thermodynamically stable association colloids of surfactant substances in which the hydrophobic radicals of the monomers lie in the interior of the aggregates and are held together by hydrophobic interaction; the hydrophilic groups face the water and by solvation provide the solubility of the colloid. Micelles occur in various shapes (spheres, rods, discs) depending on the chemical constitution of the tenside and on the temperature, concentration or ionic strength of the solution. Reaching the cmc is manifest by abrupt changes in surface tension, osmotic pressure, electrical conductivity and viscosity.

A process for preparing micelles containing ISS and antigen compositions is as follows. A micelle-forming surfactant, such as polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyoxyethylene fatty acid ester, and polyoxyethylene alkyl ethers, alkyl glycosides, is added to water at a concentration above the cmc to prepare a micellar dispersion. To the micellar dispersion is added an aqueous solution of an ISS and antigen-containing composition and the mixture is allowed to stand for a given period of time, preferably under warming at 40°C or higher, followed by cooling, to thereby prepare micelles containing ISS and antigen-containing compositions in the micellar membrane. Alternatively, the desired micelles can also be prepared by previously mixing the above-described micelle-forming substances and ISS and antigen-containing compositions and treating the mixture according to known methods for micelle formation.

In embodiments in which an ISS and antigen are proximately associated by linkage to a platform molecule, the platform may be proteinaceous or non-proteinaceous (i.e., organic). Examples of proteinaceous platforms include, but are not limited to, albumin, gammaglobulin, immunoglobulin (IgG) and ovalbumin. Borel et al. (1990) *Immunol. Methods* 126:159-168; Dumas et al. (1995) *Arch. Dermatol. Res.* 287:123-128; Borel et al.

(1995) *Int. Arch. Allergy Immunol.* 107:264-267; Borel et al. (1996) *Ann. N.Y. Acad. Sci.* 778:80-87. A platform is multi-valent (i.e., contains more than one binding, or linking, site) to accommodate binding to both an ISS and antigen. Other examples of polymeric platforms are dextran, polyacrylamide, ficoll, carboxymethylcellulose, polyvinyl alcohol, and poly D-glutamic acid/D-lysine.

5 The principles of using platform molecules are well understood in the art. Generally, a platform contains, or is derivatized to contain, appropriate binding sites for ISS and antigen. In addition, or alternatively, ISS and/or antigen is derivatized to provide appropriate linkage groups. For example, a simple platform is a bi-functional linker (i.e., 10 has two binding sites), such as a peptide. Further examples are discussed below.

Platform molecules may be biologically stabilized, *i.e.*, they exhibit an *in vivo* excretion half-life often of hours to days to months to confer therapeutic efficacy, and are preferably composed of a synthetic single chain of defined composition. They generally have a molecular weight in the range of about 200 to about 200,000, preferably about 200 15 to about 50,000 (or less, such as 30,000). Examples of valency platform molecules are polymers (or are comprised of polymers) such as polyethylene glycol (PEG; preferably having a molecular weight of about 200 to about 8000), poly-D-lysine, polyvinyl alcohol, polyvinylpyrrolidone, D-glutamic acid and D-lysine (in a ratio of 3:2). Other molecules 20 that may be used are albumin and IgG.

20 Other platform molecules suitable for use within the present invention are the chemically-defined, non-polymeric valency platform molecules disclosed in U.S. patent 5,552,391. Other homogeneous chemically-defined valency platform molecules suitable for use within the present invention are derivatized 2,2'-ethylenedioxydiethylamine (EDDA) and triethylene glycol (TEG).

25 Additional suitable valency platform molecules include, but are not limited to, tetraaminobenzene, heptaaminobetacyclodextrin, tetraaminopentaerythritol, 1,4,8,11-tetraazacyclotetradecane (Cyclam) and 1,4,7,10-tetraazacyclododecane (Cyclen).

In general, these platforms are made by standard chemical synthesis techniques. 30 PEG must be derivatized and made multivalent, which is accomplished using standard techniques. Some substances suitable for conjugate synthesis, such as PEG, albumin, and IgG are available commercially.

Conjugation of an ISS and antigen to a platform molecule may be effected in any number of ways, typically involving one or more crosslinking agents and functional groups on the antigen and ISS platform and platform molecule. Platforms and ISS and antigen must have appropriate linking groups. Linking groups are added to platforms using standard synthetic chemistry techniques. Linking groups may be added to polypeptide antigens and ISS using either standard solid phase synthetic techniques or recombinant techniques. Recombinant approaches may require post-translational modification in order to attach a linker, and such methods are known in the art.

As an example, polypeptides contain amino acid side chain moieties containing functional groups such as amino, carboxyl or sulphydryl groups that serve as sites for coupling the polypeptide to the platform. Residues that have such functional groups may be added to the polypeptide if the polypeptide does not already contain these groups. Such residues may be incorporated by solid phase synthesis techniques or recombinant techniques, both of which are well known in the peptide synthesis arts. When the polypeptide has a carbohydrate side chain(s) (or if the antigen is a carbohydrate), functional amino, sulphydryl and/or aldehyde groups may be incorporated therein by conventional chemistry. For instance, primary amino groups may be incorporated by reaction with ethylenediamine in the presence of sodium cyanoborohydride, sulphydryls may be introduced by reaction of cysteamine dihydrochloride followed by reduction with a standard disulfide reducing agent, while aldehyde groups may be generated following periodate oxidation. In a similar fashion, the platform molecule may also be derivatized to contain functional groups if it does not already possess appropriate functional groups.

Hydrophilic linkers of variable lengths are useful for connecting ISS and antigen to platform molecules. Suitable linkers include linear oligomers or polymers of ethylene glycol. Such linkers include linkers with the formula  $R^1S(CH_2CH_2O)_nCH_2CH_2O(CH_2)_mCO_2R^2$  wherein  $n = 0-200$ ,  $m = 1$  or  $2$ ,  $R^1 = H$  or a protecting group such as trityl,  $R^2 = H$  or alkyl or aryl, *e.g.*, 4-nitrophenyl ester. These linkers are useful in connecting a molecule containing a thiol reactive group such as haloacyl, maleimide, etc., via a thioether to a second molecule which contains an amino group via an amide bond. These linkers are flexible with regard to the order of attachment, *i.e.*, the thioether can be formed first or last.

In embodiments in which an ISS and antigen are proximately associated by adsorption onto a surface, the surface may be in the form of a carrier particle (for example, a nanoparticle) made with either an inorganic or organic core. Examples of such nanoparticles include, but are not limited to, nanocrystalline particles, nanoparticles made by the polymerization of alkylcyanoacrylates and nanoparticles made by the polymerization of methyldene malonate. Additional surfaces to which an ISS and antigen may be adsorbed include, but are not limited to, activated carbon particles and protein-ceramic nanoplates.

Adsorption of polynucleotides and polypeptides to a surface for the purpose of delivery of the adsorbed molecules to cells is well known in the art. See, for example, Douglas et al. (1987) *Crit. Rev. Ther. Drug. Carrier Syst.* 3:233-261; Hagiwara et al. (1987) *In Vivo* 1:241-252; Bousquet et al. (1999) *Pharm. Res.* 16:141-147; and Kossovsky et al., U.S. Patent 5,460,831. Preferably, the material comprising the adsorbent surface is biodegradable. Adsorption of an ISS and/or antigen to a surface may occur through non-covalent interactions, including ionic and/or hydrophobic interactions.

In general, characteristics of nanoparticles, such as surface charge, particle size and molecular weight, depend upon polymerization conditions, monomer concentration and the presence of stabilizers during the polymerization process (Douglas et al., 1987). The surface of carrier particles may be modified, for example, with a surface coating, to allow or enhance adsorption of the ISS and/or antigen. Carrier particles with adsorbed ISS and/or antigen may be further coated with other substances. The addition of such other substances may, for example, prolong the half-life of the particles once administered to the subject and/or may target the particles to a specific cell type or tissue, as described herein.

Nanocrystalline surfaces to which an ISS and antigen may be adsorbed have been described (see, for example, U.S. Patent 5,460,831). Nanocrystalline core particles (with diameters of 1  $\mu\text{m}$  or less) are coated with a surface energy modifying layer that promotes adsorption of polypeptides, polynucleotides and/or other pharmaceutical agents. As described in U.S. Patent 5,460,831, for example, a core particle is coated with a surface that promotes adsorption of an oligonucleotide and is subsequently coated with an antigen preparation, for example, in the form of a lipid-antigen mixture. Such nanoparticles are self-assembling complexes of nanometer sized particles, typically on the order of 0.1  $\mu\text{m}$ , that carry an inner layer of ISS and an outer layer of antigen.

Another adsorbent surface are nanoparticles made by the polymerization of alkylcyanoacrylates. Alkylcyanoacrylates can be polymerized in acidified aqueous media by a process of anionic polymerization. Depending on the polymerization conditions, the small particles tend to have sizes in the range of 20 to 3000 nm, and it is possible to make 5 nanoparticles specific surface characteristics and with specific surface charges (Douglas et al., 1987). For example, oligonucleotides may be adsorbed to polyisobutyl- and polyisohexylcyanoacrylate nanoparticles in the presence of hydrophobic cations such as tetraphenylphosphonium chloride or quaternary ammonium salts, such as cetyltrimethyl ammonium bromide. Oligonucleotide adsorption on these nanoparticles appears to be 10 mediated by the formation of ion pairs between negatively charged phosphate groups of the nucleic acid chain and the hydrophobic cations. See, for example, Lambert et al. (1998) *Biochimie* 80:969-976, Chavany et al. (1994) *Pharm. Res.* 11:1370-1378; Chavany et al. (1992) *Pharm. Res.* 9:441-449. Polypeptides may also be adsorbed to 15 polyalkylcyanoacrylate nanoparticles. See, for example, Douglas et al., 1987; Schroeder et al. (1998) *Peptides* 19:777-780.

Another adsorbent surface are nanoparticles made by the polymerization of methylidene malonate. For example, as described in Bousquet et al., 1999, polypeptides adsorbed to poly(methylidene malonate 2.1.2) nanoparticles appear to do so initially through electrostatic forces followed by stabilization through hydrophobic forces.

20 In some embodiments, the invention provides compositions comprising ISS, HIV antigen, and further comprising an adjuvant (including, but not limited to, alum, lipid emulsions, and polylactide/polyglycolide microparticles). In another embodiment, the invention provides compositions comprising ISS, an HIV antigen and an adjuvant whereby the ISS/antigen/adjuvant are co-administered. In other embodiments, the HIV antigen and 25 ISS are conjugated or proximately associated. Preferably, the immunogenic composition contains an amount of an adjuvant sufficient to potentiate the immune response to the immunogen. Preferably, adjuvants include, but are not limited to, oil-in-water emulsions, water-in oil emulsions, alum (aluminum salts), liposomes and microparticles, including but not limited to, polystyrene, starch, polyphosphazene and polylactide/polyglycosides. More 30 preferably, the ISS and antigen are co-administered with alum. More preferably, the ISS and antigen are co-administered with liposomes. Still more preferably, the ISS and antigen are co-administered with an oil-in-water emulsion. In some embodiments, the ISS and

antigen can be associated with an adjuvant through covalent and/or non-covalent interactions. An example of such non-covalent interactions includes, but is not limited to, adsorption of the ISS and antigen to microparticles described herein.

Suitable adjuvants also include, but are not limited to, squalene mixtures (SAF-1),  
5 muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875, as well as, lipid-based adjuvants and others described herein. For veterinary use and  
10 for production of antibodies in animals, mitogenic components of Freund's adjuvant (both complete and incomplete) can be used.

***Methods of modulating immune responses with ISS and antigen***

The present invention also provides methods for the use of any of the compositions described herein (generally, ISS in conjunction with an HIV antigen) in stimulation of an immune response, preferably an HIV-antigen specific immune response. Preferably, as used in such methods, the ISS provides an adjuvant-like activity in the generation of a Th1-type immune response to the antigen. The invention also provides methods of treating an individual in need of immune modulation comprising administration of an immunomodulatory composition comprising an immunomodulatory oligonucleotide and an HIV antigen, including, but not limited to, individuals infected with HIV or at risk of being infected with HIV. As the term "treatment" indicates to one skilled in the art, treating individuals as described herein can encompass any of the following: alleviation of one or more symptoms associated with HIV infection; diminishment of extent of HIV infection  
20 (including any disease that would arise due to HIV infection); stabilized condition; delay or slowing of progression; amelioration or palliation of any disease state associated with HIV infection (including AIDS); suppressing HIV infection.  
25

Accordingly, the invention provides methods in which administration of ISS-containing polypeptide and HIV antigen described herein is used to modulate an antigen-specific immune response in an individual, in particular an HIV-specific response. In some embodiments, the modulation of the immune response is by induction or increase of a Th1 response. In some embodiments, the invention provides methods of preventing and/or

delaying development of HIV infection. In some embodiments, the invention provides methods of ameliorating or palliating HIV infection, including any symptom of or associated with HIV infection. In some embodiments, the invention provides methods of suppressing HIV infection. In some embodiments, the invention provides methods of stimulating a specific immune response against an HIV antigen, preferably against gp120. 5 All such methods may be particularly beneficial to individuals infected with, or at risk of infection with, HIV.

In some embodiments, the invention provides methods of stimulating a specific immune response against an HIV antigen (which have been listed above) comprising 10 administering an ISS-containing polynucleotide and the HIV antigen in an amount sufficient to stimulate an immune response against the administered HIV antigen. Preferably, the HIV antigen is a gp120 polypeptide (including gp120). Accordingly, the invention provides methods of stimulating a specific immune response against gp120 in an individual comprising administering an ISS-containing polynucleotide and a gp120 15 polypeptide in an amount sufficient to stimulate an anti-gp120 immune response (i.e., a gp120-specific immune response). The immune response may comprise production of anti-gp120 antibody and/or production of gp120-specific T cells, especially gp120-specific cytotoxic T cells (CTLs). The immune response may also comprise stimulation of Th1 cells (including production of cytokines associated with stimulation of Th1 cells). Example 20 1 illustrates stimulating both a gp120 specific antibody response and a gp120-specific CTL response, as well as a Th1 mediated response, based on administration to a mammal (mouse) a conjugate of gp120 and SEQ ID NO:1.

For these embodiments, the ISS-containing polynucleotide and HIV antigen may be administered as an admixture (i.e., not proximately associated) or proximately associated.

25 Preferably, the ISS-containing polynucleotide and HIV antigen (such as gp120) are administered proximately associated such that the immune response is enhanced as compared to administering ISS-containing polynucleotide and HIV antigen as an admixture. In some embodiments, ISS-containing polynucleotide and gp120 are proximately associated, which includes any of the above-described embodiments for 30 proximate association, including conjugation. In some embodiments, the ISS-containing polynucleotide is SEQ ID NO:1, although any of the ISS described herein may be used.

Preferably, the immune response stimulated according to the invention is biased toward the Th1-type phenotype and away from the Th2-type phenotype. With reference to the invention, stimulating a Th1-type immune response can be determined *in vitro* or *ex vivo* by measuring cytokine production from cells treated with ISS as compared to those treated without ISS. Methods to determine the cytokine production of cells include those methods described herein and any known in the art. The type of cytokines produced in response to ISS treatment indicate a Th1-type or a Th2-type biased immune response by the cells. As used herein, the term "Th1-type biased" cytokine production refers to the measurable increased production of cytokines associated with a Th1-type immune response in the presence of a stimulator as compared to production of such cytokines in the absence of stimulation. Examples of such Th1-type biased cytokines include, but are not limited to, IL-2, IL-12, and IFN- $\gamma$ . In contrast, "Th2-type biased cytokines" refers to those associated with a Th2-type immune response, and include, but are not limited to, IL-4, IL-5, IL-10 and IL-13. Cells useful for the determination of ISS activity include cells of the immune system, primary cells isolated from a host and/or cell lines, preferably APCs and lymphocytes, even more preferably macrophages and T cells.

Stimulating a Th1-type immune response can also be measured in a host treated with an ISS-antigen composition and can be determined by any method known in the art including, but not limited to: (1) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 in an ISS-antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS; (2) an increase in levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) in an ISS-antigen treated host as compared to an antigen-primed or, primed and challenged, control treated without ISS; (3) IgG2a antibody production in an ISS-antigen treated host as compared to a control treated without ISS; and/or (4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in an ISS-antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS. A variety of these determinations can be made by measuring cytokines made by APCs and/or lymphocytes, preferably macrophages and/or T cells, *in vitro* or *ex vivo* using methods described herein

or any known in the art. Methods to determine antibody production include any known in the art.

The Th1-type biased cytokine induction which occurs as a result of ISS administration produces enhanced cellular immune responses, such as those performed by 5 NK cells, cytotoxic killer cells, Th1 helper and memory cells. These responses are particularly beneficial for use in protective or therapeutic vaccination against viruses, fungi, protozoan parasites, bacteria, allergic diseases and asthma, as well as tumors.

For all of the methods described herein, an effective amount of ISS-containing polynucleotide and HIV antigen is administered, that is, an amount sufficient to achieve the 10 desired result(s), depending on the objectives of treatment. An effective amount may be given in single or divided doses (i.e., in one or more administrations). Generally, if the ISS-containing polynucleotide and HIV antigen are administered in proximate association to each other (such as in a conjugate form), a dosage range of the ISS-antigen composition may be, for example, from about any of the following: 1 to 500 µg, 100 to 400 µg, 200 to 15 300 µg, 1 to 100 µg, 100 to 200 µg, 300 to 400 µg, 400 to 500 µg. In these compositions, the molar ratio of ISS-containing polynucleotide to antigen may vary. If the ISS-containing polynucleotide and HIV antigen are not proximately associated, i.e., administered as an admixture, generally the dosage ranges are higher, such as, for the ISS-containing 20 polynucleotide, for example, from about any of the following: 10 to 10,000 µg, 2000 to 8000 µg, 4000 to 6000 µg, 10 to 500 µg, 500 to 1000 µg, 1000 to 2000 µg, 2000 to 3000 µg, 6000 to 7000 µg, 7000 to 8000 µg, 8000 to 9000 µg, 9000 to 10,000 µg; and for the 25 HIV antigen, for example, from about any of the following: 0.1 to 500 µg, 1.0 to 100 µg, 5 to 50 µg, 0.1 to 1.0 µg, 1.0 to 10 µg, 50 to 200 µg, 200 to 400 µg, 300 to 500 µg.

The effectiveness of ISS/HIV antigen administration can be monitored by several 25 criteria. Assays for cytokines (including Th1-associated cytokines) have been described above and herein, and are known in the art. Other assays for an immune response are also known in the art, such as assays for antibodies (such as ELISA) and T cells. As is well known in the art, the treated subject may also be monitored for clinical features which accompany the HIV infection. For example, subjects may be monitored for presence and/or levels of p24 30 protein (which indicates amount of virus present and can also indicate degree of neutralization of virus); and reduction in a symptom associated with infection (such as incidence of opportunistic infections). Individuals may also be monitored for levels of T helper

populations, wherein an increase indicates an effective amount has been administered. For example, after treatment, the individual may be assessed for improvement in a number of clinical parameters including, but not limited to, reduction of incidence and/or symptoms of opportunistic infections. An opportunistic infection, as is well known in the art, may be any infection (whether viral or bacterial) that arise from immunosuppression due to HIV infection. For methods in which HIV infection is suppressed, any indicator of HIV infection, such as presence of HIV RNA, HIV DNA, T cell count, presence of neutralizing anti-HIV antibodies (as measured by p24 levels) or HIV antigen, or symptom associated with HIV infection may be checked and/or monitored using methods known in the art. For methods in which a symptom associated with HIV infection is ameliorated, any such symptom may be checked and/or monitored to confirm that an effective amount of ISS-containing polynucleotide and HIV antigen have been administered. For methods in which HIV infection is palliated or treated, any indicia of HIV infection (such as those listed above) may be checked and/or monitored that an effective amount has been administered.

The selection of a particular composition, dosage regimen (*i.e.*, dose, timing and repetition) and route of administration will depend on a number of different factors, including, but not limited to, the subject's medical history and features of the condition and the subject being treated. The assessment of such features and the design of an appropriate therapeutic regimen is ultimately the responsibility of the prescribing physician. The particular dosage regimen may be determined empirically.

*Individuals receiving ISS and HIV antigen*

Individuals suitable for receiving ISS-containing polynucleotide and antigen include those who have been infected by HIV as well as those who are at risk of being infected by and/or exposed to HIV. Infection by HIV is indicated by any suitable assay or measurable parameter, such as detection of HIV DNA in a clinical (*i.e.*, biological) sample; detection of number of T helper cells; detection of an HIV antigen or anti-HIV antibody; one or more symptoms associated with HIV infection, such as any symptom associated with an opportunistic infection. Such detection systems and clinical parameters are known in the art.

Another type of individual suitable for receiving ISS-containing polynucleotide and HIV antigen are those individuals at risk for infection with HIV. These risk groups, known

in the art, include, for example, individuals who engage in so-called "unprotected" sexual relations; individuals who are intravenous drug users; and health care workers.

*Administration and assessment*

ISS may be administered with HIV antigen in a number of ways. ISS-containing polynucleotide(s) and HIV antigen may be administered as an admixture (i.e., in solution) or, more preferably, be administered spatially proximate with respect to each other, whether by conjugation or by other means of effecting spatial proximation, which have been described above.

The ISS-containing polynucleotide may be any of those described above. Preferably, the ISS-containing polynucleotide administered comprises the formula 5' purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'; more preferably, 5'-A, A, C, G, T, T, C, G-3'. Another preferred embodiment uses SEQ ID NO:1.

The ISS and the HIV antigen(s) can be administered together in the form of a conjugate or co-administered in an admixture sufficiently close in time so as to modulate an immune response. Preferably, the ISS and antigen are administered simultaneously. The term "co-administration" as used herein refers to the administration of at least two different substances sufficiently close in time to modulate an immune response. Preferably, co-administration refers to simultaneous administration of at least two different substances.

The ISS and antigen can be administered as an ISS-antigen conjugate and/or they can be co-administered as a complex in the form of an admixture, such as in an emulsion, and/or administered in spatial proximation such that the immune response is enhanced as compared to administering in a solution. As described above, the association of the ISS and the antigen molecules in an ISS-antigen conjugate can be through covalent interactions and/or through non-covalent interactions, including high affinity and/or low affinity interactions. Examples of non-covalent interactions that can couple an ISS and an antigen in an ISS-antigen conjugate include, but are not limited to, ionic bonds, hydrophobic interactions, hydrogen bonds and van der Waals attractions.

In another embodiment, ISS and antigen can be administered in conjunction with one or more immunomodulatory facilitator. Thus, the invention provides compositions comprising ISS and an immunomodulatory facilitator. As used herein, the term "immunomodulatory facilitator" refers to molecules which support and/or enhance the immunomodulatory activity of an ISS and/or an antigen. Examples of immunomodulatory

facilitators can include co-stimulatory molecules, such as cytokines, and/or adjuvants. The ISS, antigen and facilitator can be administered as an ISS-antigen-facilitator conjugate and/or they can be co-administered as a complex in the form of an admixture, such as in an emulsion. The association of the ISS, the antigen and the facilitator molecules in an ISS-  
5 antigen-facilitator conjugate can be through covalent interactions and/or through non-covalent interactions, including high affinity and/or low affinity interactions. Examples of non-covalent interactions that can couple an ISS, an antigen and a facilitator in an ISS-antigen-facilitator conjugate include, but are not limited to, ionic bonds, hydrophobic interactions, hydrogen bonds and van der Waals attractions.

10 Immunomodulatory facilitators include, but are not limited to, co-stimulatory molecules (such as cytokines, chemokines, targeting protein ligand, trans-activating factors, peptides, and peptides comprising a modified amino acid) and adjuvants (such as alum, lipid emulsions, and polylactide/polyglycolide microparticles).

15 Among suitable immunomodulatory cytokine peptides for administration with ISS and antigen are the interleukins (e.g., IL-1, IL-2, IL-3, etc.), interferons (e.g., IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ ), erythropoietin, colony stimulating factors (e.g., G-CSF, M-CSF, GM-CSF) and TNF- $\alpha$ . Preferably, immunostimulatory peptides for use in conjunction with ISS and antigen are those that stimulate Th1-type immune responses, such as IL-12 (Bliss et al.  
20 (1996) *J. Immunol.* 156:887-894), IL-18, TNF- $\alpha$ ,  $\beta$  and  $\gamma$ , and/or transforming growth factor (TGF)- $\alpha$ .

25 Peptides administered with ISS and antigen can also include amino acid sequences that mediate protein binding to a specific receptor or that mediate targeting to a specific cell type or tissue. Examples include, but are not limited to, antibodies or antibody fragments, peptide hormones such as human growth hormone, and enzymes. Immunomodulatory peptides also include peptide hormones, peptide neurotransmitters and peptide growth factors. Co-stimulatory molecules such as B7 (CD80), trans-activating proteins such as transcription factors, chemokines such as macrophage chemotactic protein (MCP) and other chemoattractant or chemotactic peptides are also useful peptides for administration with ISS and antigen.

30 The invention also provides for the administration of ISS and antigen in conjunction with an adjuvant. Administration of an antigen with an ISS and an adjuvant leads to a potentiation of a immune response to the antigen and thus, can result in an enhanced

immune response compared to that which results from a composition comprising the ISS and antigen alone. For example, we have shown that administration of an antigen with an ISS and an adjuvant leads to an enhanced primary immune response. Examples of suitable adjuvants have been discussed above.

5 As with all immunogenic compositions, the immunologically effective amounts of the components must be determined empirically. Factors to be considered include the antigenicity, whether or not ISS and/or antigen will be complexed with or covalently attached to an immunomodulatory facilitator, an adjuvant or carrier protein or other carrier, route of administration and the number of immunizing doses to be administered. Such 10 factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

15 The compositions of the present invention can be administered alone or in combination with other pharmaceutical and/or immunogenic and/or immunostimulatory agents and can be combined with a physiologically acceptable carrier thereof. The effective amount and method of administration of the particular ISS and antigen 20 formulation can vary based on the individual patient and the stage of the disease and other factors evident to one skilled in the art. The route(s) of administration useful in a particular application are apparent to one of skill in the art. Routes of administration include but are not limited to topical, dermal, transdermal, transmucosal, epidermal, parenteral, gastrointestinal, and naso-pharyngeal and pulmonary, including transbronchial and transalveolar. A suitable dosage range is one that provides sufficient ISS-containing composition to attain a tissue concentration of about 1-10  $\mu$ M as measured by blood levels. The absolute amount given to each patient depends on pharmacological properties such as bioavailability, clearance rate and route of administration.

25 As described herein, APCs and tissues with high concentration of APCs are preferred targets for the ISS and antigen-containing compositions. Thus, administration of ISS and antigen-containing compositions to mammalian skin and/or mucosa, where APCs are present in relatively high concentration, is preferred.

30 The present invention provides ISS and antigen-containing compositions suitable for topical application including, but not limited to, physiologically acceptable implants, ointments, creams, rinses and gels. Topical administration is, for instance, by a dressing or bandage having dispersed therein a delivery system, or by direct administration of a

delivery system into incisions or open wounds. Creams, rinses, gels or ointments having dispersed therein an ISS and antigen-containing composition are suitable for use as topical ointments or wound filling agents.

Preferred routes of dermal administration are those which are least invasive.

5 Preferred among these means are transdermal transmission, epidermal administration and subcutaneous injection. Of these means, epidermal administration is preferred for the greater concentrations of APCs expected to be in intradermal tissue.

10 Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the ISS and antigen-containing composition to penetrate the skin and enter the blood stream. Compositions suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference.

15 For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission can be accomplished using commercially available patches which deliver their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for 20 contemporaneous use of an absorption promoter.

25 An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

30 For transdermal transmission, low-frequency ultrasonic delivery is also a suitable method. Mitragotri et al. (1995) *Science* 269:850-853. Application of low-frequency ultrasonic frequencies (about 1 MHz) allows the general controlled delivery of therapeutic compositions, including those of high molecular weight.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APCs to the site of irritation.

5 An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tines which can be used to irritate the skin and attract APCs to the site of irritation, to take up ISS and antigen-containing compositions transferred from the end of the tines. For example, the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France contains a device suitable for introduction of ISS and antigen-containing compositions.

10 The device (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, PA) consists of a plastic container having a syringe plunger at one end and a tine disk at the other. The tine disk supports a multiplicity of narrow diameter tines of a length which will just scratch the outermost layer of epidermal cells. Each of the tines in 15 the MONO-VACC kit is coated with old tuberculin; in the present invention, each needle is coated with a pharmaceutical composition of ISS and antigen-containing composition. Use of the device is preferably according to the manufacturer's written instructions included with the device product. Similar devices which can also be used in this embodiment are those which are currently used to perform allergy tests.

20 Another suitable approach to epidermal administration of ISS and antigen-containing compositions is by use of a chemical which irritates the outermost cells of the epidermis, thus provoking a sufficient immune response to attract APCs to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR. 25 This approach can also be used to achieve epithelial administration in the mucosa. The chemical irritant can also be applied in conjunction with the mechanical irritant (as, for example, would occur if the MONO-VACC type tine were also coated with the chemical irritant). The ISS and antigen-containing compositions can be suspended in a carrier which also contains the chemical irritant or coadministered therewith.

30 Another delivery method for administering ISS and antigen-containing compositions makes use of non-lipid polymers, such as a synthetic polycationic amino polymer. Leff (1997) *Bioworld* 86:1-2.

Parenteral routes of administration include, but are not limited to, electrical (iontophoresis) or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Compositions suitable for parenteral administration include, but are not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for injection of the ISS and antigen-containing compositions.

Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. The invention includes ISS and antigen-containing compositions suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable, powders, pills or liquids for ingestion and suppositories for rectal administration.

In some embodiments, the ISS-containing polynucleotide and HIV antigen are administered via mucosal immunization (i.e., administration to the mucosa). Mucosal immunization may be by any of a number of means, such as by topical application to the mucosa (including by spray or drops) or injection into the mucosa, in addition to those described below.

Naso-pharyngeal and pulmonary routes of administration include, but are not limited to, by-inhalation, transbronchial and transalveolar routes. The invention includes ISS and antigen-containing compositions suitable for by-inhalation administration including, but not limited to, various types of aerosols for inhalation, as well as powder forms for delivery systems. Devices suitable for by-inhalation administration of ISS and antigen-containing compositions include, but are not limited to, atomizers and vaporizers. Atomizers and vaporizers filled with the powders are among a variety of devices suitable for use in by-inhalation delivery of powders. See, e.g., Lindberg (1993) Summary of Lecture at Management Forum 6-7 December 1993 "Creating the Future for Portable Inhalers."

The methods of producing suitable devices for injection, topical application, atomizers and vaporizers are known in the art and will not be described in detail.

The choice of delivery routes can be used to modulate the immune response elicited. For example, IgG titers and CTL activities were identical when an influenza virus vector was administered via intramuscular or epidermal (gene gun) routes; however, the muscular inoculation yielded primarily IgG2a, while the epidermal route yielded mostly

IgG1. Pertmer et al. (1996) *J. Virol.* 70:6119-6125. Thus, one of skill in the art can take advantage of slight differences in immunogenicity elicited by different routes of administering the immunomodulatory compositions of the present invention.

Analysis (both qualitative and quantitative) of the immune response to ISS and antigen-containing compositions can be by any method known in the art, including, but not limited to, measuring antigen-specific antibody production, activation of specific populations of lymphocytes such as CD4<sup>+</sup> T cells or NK cells, and/or production of cytokines such as IFN, IL-2, IL-4, or IL-12. Methods for measuring specific antibody responses include enzyme-linked immunosorbent assay (ELISA) and are well known in the art. Measurement of numbers of specific types of lymphocytes such as CD4<sup>+</sup> T cells can be achieved, for example, with fluorescence-activated cell sorting (FACS). Cytotoxicity assays are known in the art and can be performed, for instance, as described in Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523. Serum concentrations of cytokines can be measured, for example, by ELISA. These and other assays to evaluate the immune response to an immunogen are well known in the art. See, for example, *Selected Methods in Cellular Immunology* (1980) Mishell and Shiigi, eds., W.H. Freeman and Co.

The above-mentioned compositions and methods of administration are meant to describe but not limit the methods of administering the ISS and antigen-containing compositions of the invention. The methods of producing the various compositions and devices are within the ability of one skilled in the art and are not described in detail here.

The following examples are provided to illustrate, but not limit, the invention.

#### EXAMPLES

25

##### EXAMPLE 1

###### Selective Induction of a Th1-type Response in a Host after Administration of a Composition Comprising an ISS and antigen

As described herein, a Th1-type immune response is associated with the production of specific cytokines, such as IFN- $\gamma$ , and results in production of CTLs.

To determine if a Th1-type immune response would be produced in mice receiving ISS and antigen compositions according to the invention, mice were immunized with HIV

gp120 protein, with and without co-administration of ISS oligonucleotides. The ISS and gp120 were administered in the form of an ISS-gp120 conjugate or as an admixture in saline. A set of mice received gp120 without ISS oligonucleotide.

*Conjugation of oligonucleotide with HIV gp120*

5 Phosphorothioate ISS oligonucleotides were coupled to thiol groups on the protein by the following technique. Recombinant gp120, expressed in CHO cells, was obtained from Quality Biologicals, Inc. Five hundred micrograms of gp120 (4.2 nmole) was treated with 20-50 fold excess of sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate) at room temperature for 2 hours. The maleimido-modified 10 gp120 was purified from unreacted sulfo-SMCC by filtration through a NAP-25 column. A 5'-disulfide modified ISS oligonucleotide (Glen Research, 5'-Amino Modifier 6) was reduced to the thiol by treatment with tricarboxyethyl phosphine (TCEP) in PBS. Following 15 purification by gel filtration and concentration by diafiltration, 10-30 molar excess of the thiol-activated oligonucleotide was incubated with the maleimido-modified gp120 at room temperature over night. Free oligonucleotide was removed and the oligonucleotide-gp120 15 conjugate was concentrated using spin columns.

The conjugate was analyzed, after electrophoresis on a 4-20% SDS-PAGE gel, by UV-shadowing, CB-staining and immunoblotting with an anti-gp120 antibody. All of these analyses demonstrated successful conjugation of the protein and the oligonucleotide. 20 Quantitation of the oligonucleotide/protein molar ratio was determined by a UV assay comparing the conjugate to standards composed of gp120 and molar ratios of the oligonucleotide.

25 The ISS oligonucleotide used in the conjugate was prepared by Trilink BioTechnologies (San Diego, CA) and has the sequence 5'-TGACTGTGAACGTTGAGATGA-3' [SEQ ID NO:1].

*Immunization and Immune Response*

Generally, for these experiments, groups of four, eight week old female BALB/c mice were immunized once intradermally with 10 µg ISS-gp120 conjugate, 10 µg ISS 30 mixed with gp120 in saline, or 10 µg gp120. Mice were bled every two weeks post-immunization, serum separated and stored at -20°C for later analysis. Three days prior to sacrifice, mice were injected intravenously with 5 µg gp120. Naïve mice are also included in the experiments.

The cytokine secretion profile, antigen-dependent antibody responses and antigen-dependent CTL responses of these mice were tested *in vitro*.

Cytokine secretion was determined by ELISA tests. Generally, to measure cytokine secretion, splenocytes were harvested and resuspended in RP10 medium at  $5 \times 10^5$  5 splenocytes per well in 96 well flat-bottomed tissue culture microtiter plates. Culture medium alone as control or gp120 protein was added to triplicate wells. Culture supernatants were sampled at 48 and 72 hours and then analyzed by ELISA for cytokine levels. The results of such an experiment are depicted in Figure 1. Cells from mice injected with the ISS-gp120 conjugate secreted IFN- $\gamma$ , a cytokine associated with a Th1-type immune response. Cells from mice injected with gp120 mixed with ISS in saline and 10 cells from mice injected with gp120 alone did not secrete detectable levels of IFN- $\gamma$ .

Anti-gp120 antibodies of the IgG2a and IgG1 isotypes were measured by ELISA as described in Raz et al. (1996) and Sato et al. (1996). IgG2a and IgG1 results from such an experiment are depicted in Figure 2 and 3, respectively. As shown in Figure 2, 15 administration of gp120 alone or in a mixture with ISS resulted in almost no anti-gp120 IgG2a production, whereas administration of an ISS-gp120 conjugate generated a significant level of anti-gp120 IgG2a antibody. As described herein, the IgG2a antibody isotype is indicative of a Th1-type immune response.

gp120-specific CTL responses were determined as described in Sato et al. (1996). 20 As depicted in Figure 4, the cells from mice that received ISS-gp120 conjugate had very strong anti-gp120 CTL activity. Cells from mice injected with gp120 mixed with ISS in saline and cells from mice injected with gp120 alone had very little CTL activity.

Serum from the injected mice were also tested for the presence of HIV neutralizing 25 antibodies by the determination of HIV p24 levels. Results of such an experiment are depicted in Figure 5.

CLAIMS

We claim:

1. An immunomodulatory composition comprising an human immunodeficiency virus (HIV) antigen and further comprising an immunomodulatory polynucleotide, wherein said polynucleotide comprises an immunostimulatory sequence (ISS).  
5
2. The immunomodulatory composition of claim 1, wherein the immunomodulatory polynucleotide comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.  
10
3. The immunomodulatory composition of claim 1, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.
- 15 4. The immunomodulatory composition of claim 1, wherein the antigen is an HIV gp120 polypeptide.
5. An immunomodulatory composition according to claim 4, wherein the immunomodulatory polynucleotide is SEQ ID NO:1 and wherein the antigen is HIV gp120.  
20
6. The immunomodulatory composition of claim 1, wherein the HIV antigen is conjugated to the immunomodulatory polynucleotide.
- 25 7. The immunomodulatory composition of claim 6, wherein the HIV antigen is a gp120 polypeptide.
8. The immunomodulatory composition of claim 7, wherein the HIV antigen is gp120 and the immunomodulatory polynucleotide comprises SEQ ID NO:1.
- 30 9. The immunomodulatory composition of claim 1, wherein the HIV antigen is not conjugated to the immunomodulatory polynucleotide and is proximately associated to the immunomodulatory polynucleotide and at a distance such that an immune response is

enhanced compared to co-administration of the immunomodulatory polynucleotide and antigen in solution.

10. The immunomodulatory composition of claim 9, wherein the HIV antigen is a gp120

5 polypeptide.

11. The immunomodulatory composition of claim 10, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

10 12. A method of modulating an immune response comprising administration of an immunomodulatory composition according to claim 1 in an amount sufficient to modulate the immune response.

15 13. The method of claim 12, wherein the modulating of an immune response comprises induction of a Th1 response.

14. The method of claim 12, wherein the immunomodulatory polynucleotide and HIV antigen are co-administered.

20 15. The method of claim 12, wherein the immunomodulatory polynucleotide and HIV antigen are conjugated.

16. The method of claim 12, wherein the immunomodulatory polynucleotide and HIV antigen are not conjugated and are proximately associated at a distance such that an 25 immune response is enhanced compared to co-administration of the immunomodulatory polynucleotide and antigen in solution.

17. The method of claim 15, wherein the HIV antigen is a gp120 polypeptide.

30 18. The method of claim 17, wherein the HIV antigen is gp120.

19. The method of claim 18, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

20. The method of claim 16, wherein the HIV antigen is a gp120 polypeptide.

5

21. The method of claim 20, wherein wherein the HIV antigen is gp120.

22. The method of claim 21, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

10

23. The method of claim 17, wherein the modulating of an immune response comprises induction of a Th1 response.

15

24. The method of claim 20, wherein the modulating of an immune response comprises induction of a Th1 response.

20

25. A method of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120 polypeptide and an immunomodulatory polynucleotide, wherein said polynucleotide comprises an immunostimulatory sequence (ISS), wherein the gp120 polypeptide is conjugated to the immunomodulatory polynucleotide, and wherein the immunomodulatory composition is administered to the individual in an amount sufficient to stimulate a specific immune response against gp120.

25

26. The method of claim 25, wherein the polypeptide is gp120.

27. The method of claim 26, wherein gp120 is conjugated to the immunomodulatory polynucleotide.

30

28. The method of claim 27, wherein the immunomodulatory polynucleotide comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

29. The method of claim 28, wherein the immunomodulatory polynucleotide comprises SEQ ID NO:1.

30. The method of claim 25, wherein the specific immune response comprises production  
5 of anti-gp120 antibody.

31. The method of claim 25, wherein the specific immune response comprises production  
of gp120-specific T cells.

10 32. The method of claim 31, wherein the gp120-specific T cells are cytotoxic T cells.

33. A method of stimulating a specific immune response against gp120 in an individual, comprising administering an immunomodulatory composition comprising a gp120  
5 polypeptide and an immunomodulatory polynucleotide, wherein said polynucleotide  
comprises an immunostimulatory sequence (ISS), wherein the gp120 polypeptide is not  
conjugated and is proximately associated to the immunomodulatory polynucleotide at a  
distance such that an immune response is enhanced compared to co-administration of the  
15 immunomodulatory polynucleotide and gp120 polypeptide in solution, and wherein the  
immunomodulatory composition is administered to the individual in an amount sufficient  
to stimulate a specific immune response against gp120.

20 34. The method of claim 33, wherein the polypeptide is gp120.

35. The method of claim 34, wherein gp120 is conjugated to the immunomodulatory  
25 polynucleotide.

36. The method of claim 35, wherein the immunomodulatory polynucleotide comprises the  
sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

30 37. The method of claim 36, wherein the immunomodulatory polynucleotide comprises  
SEQ ID NO:1.

38. The method of claim 33, wherein the specific immune response comprises production of anti-gp120 antibody.

39. The method of claim 33, wherein the specific immune response comprises production 5 of gp120-specific T cells.

40. The method of claim 29, wherein the gp120-specific T cells are cytotoxic T cells.

41. A method of suppressing HIV infection in an individual infected with HIV, comprising 10 administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection.

42. A method of delaying development of HIV infection in an individual at risk of 15 infection with HIV, comprising administering to the individual a composition comprising gp120 conjugated to an immunomodulatory oligonucleotide consisting of SEQ ID NO:1 in an amount sufficient to suppress HIV infection upon exposure to HIV.

43. A method of treating an individual in need of immune modulation, wherein the 20 individual has been infected with HIV, comprising administration of the immunomodulatory composition of claim 1 to the individual.

1/5

Figure 1

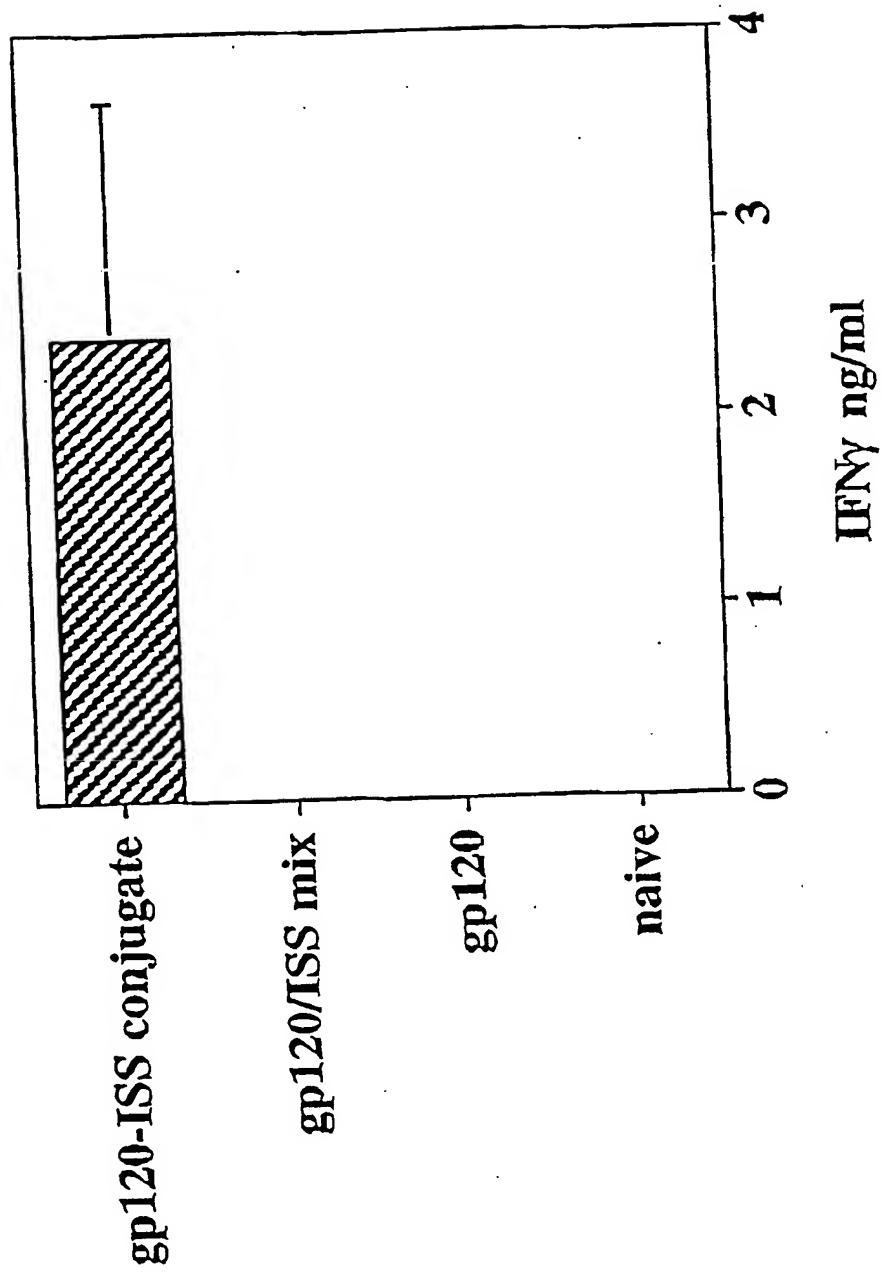
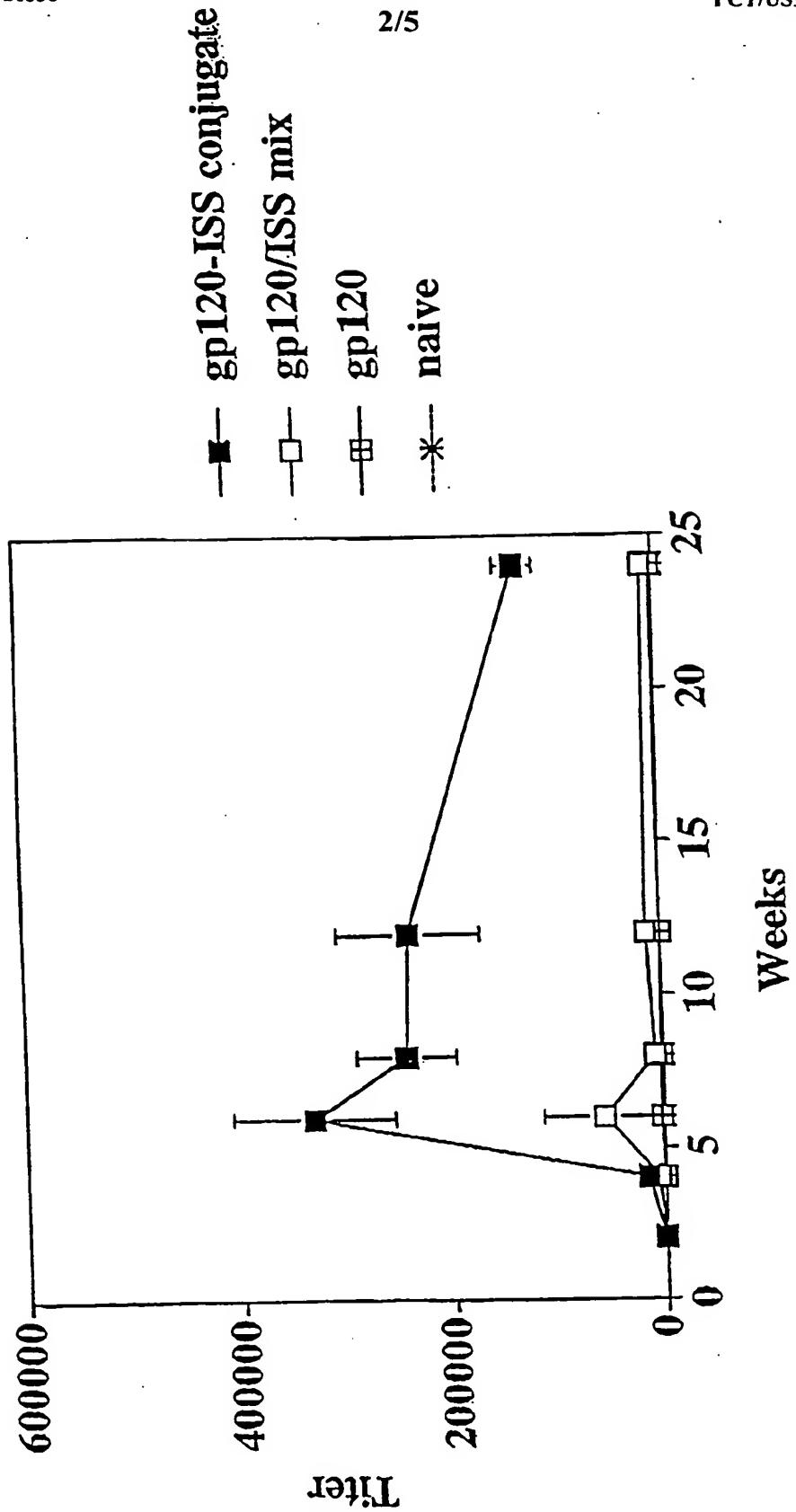


Figure 2



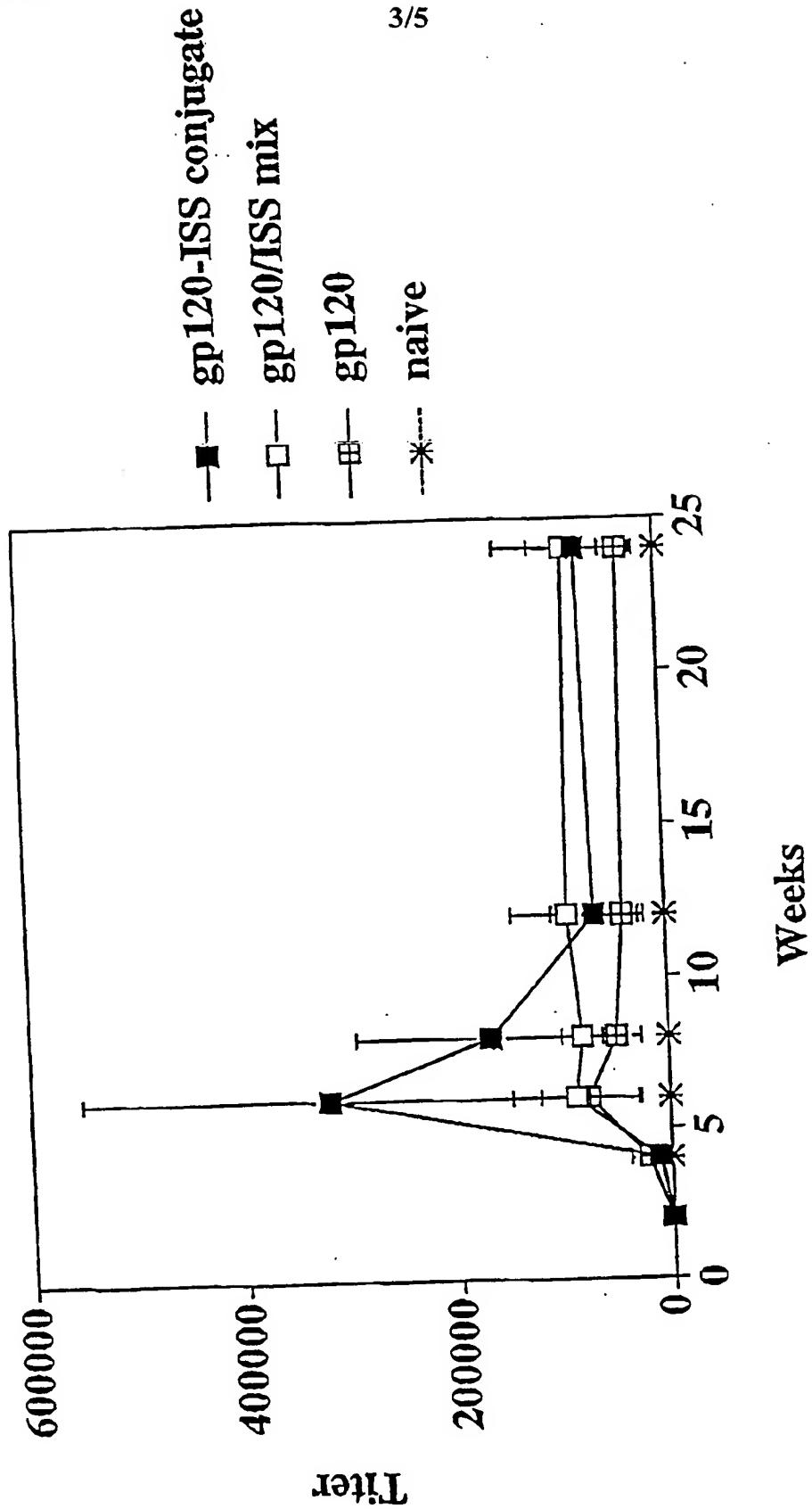
**Figure 3**

Figure 4

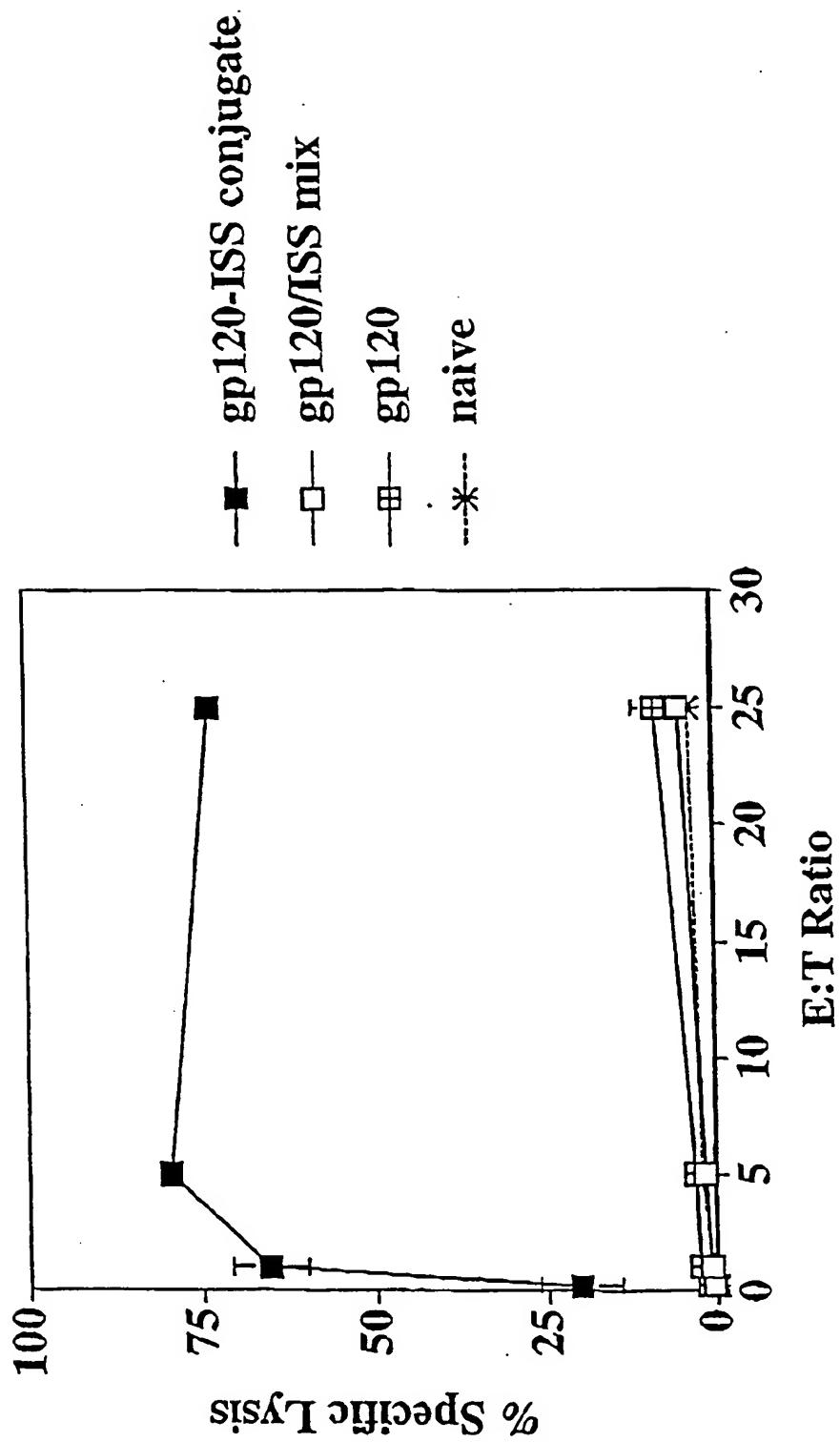
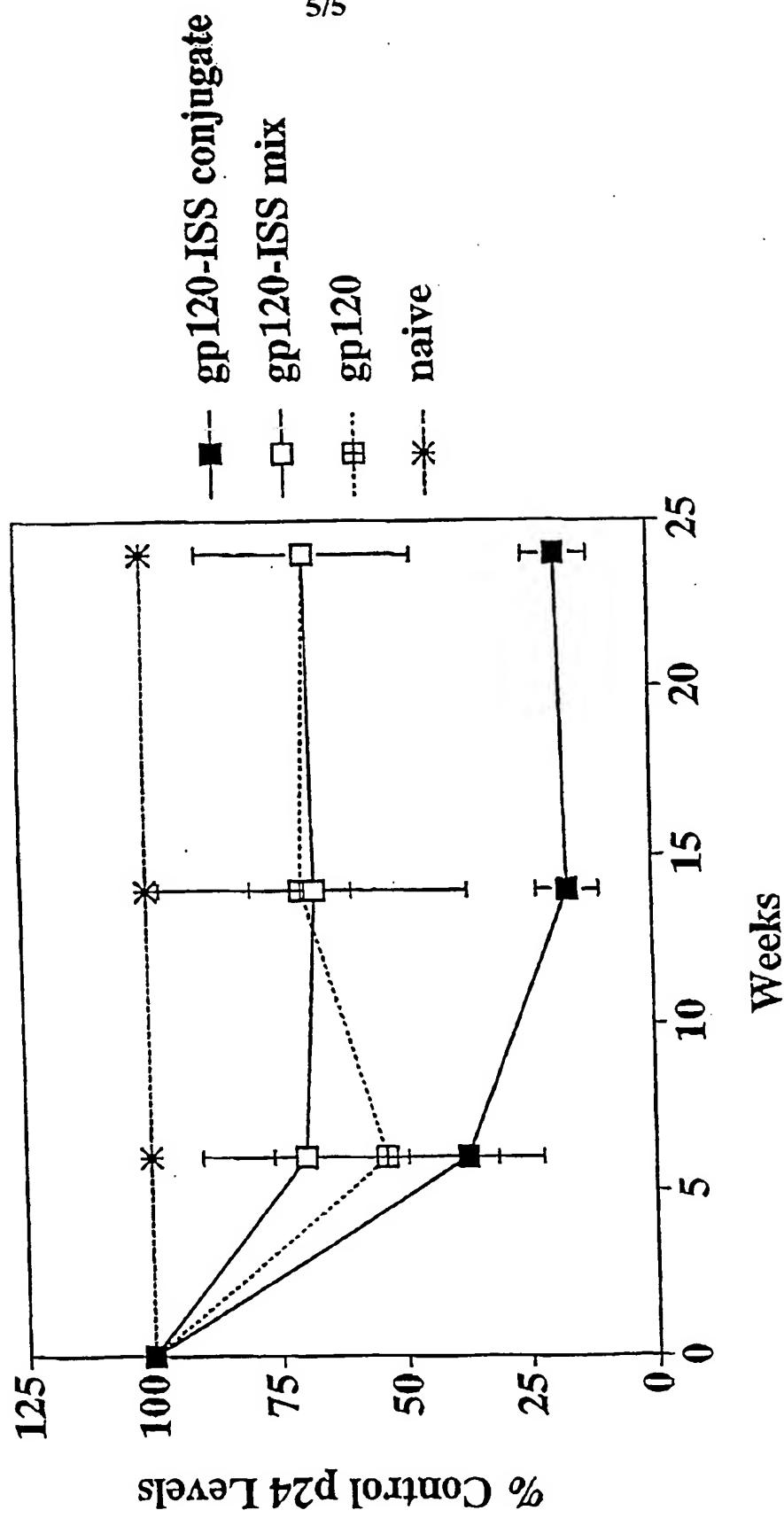


Figure 5



# INTERNATIONAL SEARCH REPORT

Inte...nal Application No  
PCT/US 99/23677

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 16247 A (CARSON DENNIS A ; RAZ EYAL (US); ROMAN MARK (US); UNIV CALIFORNIA ()) 23 April 1998 (1998-04-23) the whole document, especially page 15 lines 1-3; page 18 lines 7-12; page 35-36 example 1; claims 1, 66 ---	1-43
X, P, L	WO 98 55495 A (DYNAVAX TECHNOLOGIES CORP ; DINA DINO (US); ROMAN MARK (US); SCHWAR) 10 December 1998 (1998-12-10) the whole document ---	1-43
E	WO 99 51259 A (UNIV IOWA RES FOUND) 14 October 1999 (1999-10-14) the whole document ---	1, 12-14, 16, 43 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

10 March 2000

Date of mailing of the International search report

28/03/2000

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## INTERNATIONAL SEARCH REPORT

Int'l. Application No

PCT/US 99/23677

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	DEML L ET AL: "IMMUNOSTIMULATORY CPG MOTIFS TRIGGER A T HELPER-1 IMMUNE RESPONSE TO HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) GP160 ENVELOPE PROTEINS" CLIN CHEM LAB MED, vol. 37, no. 3, March 1999 (1999-03), pages 199-204-204, XP000857051  the whole document ----	1,2,4,5, 7,9,10, 12-18, 20,21, 23-28, 30-34, 36, 38-40,43
A	WO 98 18810 A (UNIV IOWA RES FOUND ;KLINE JOEL N (US); KRIEG ARTHUR M (US)) 7 May 1998 (1998-05-07) the whole document ----	1-43
A	MICHAEL F. POWELL AT AL: "Vaccine Design- The subunit and adjuvant approach" 1996 , PLENUM PRESS , NEW YORK XP002132738 page 821 -page 845 ----	1-43

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/23677

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 12-43 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2.  Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3.  Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Intern. Appl. No.

PCT/US 99/23677

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9816247	A 23-04-1998	AU 4992197 A		11-05-1998
		EP 0930893 A		28-07-1999
WO 9855495	A 10-12-1998	AU 7811398 A		21-12-1998
		AU 7817898 A		21-12-1998
		WO 9855609 A		10-12-1998
WO 9951259	A 14-10-1999	AU 3467899 A		25-10-1999
WO 9818810	A 07-05-1998	AU 5242498 A		22-05-1998
		CN 1235609 A		17-11-1999
		EP 0948510 A		13-10-1999

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